Universidad Politécnica de Cartagena
Departamento de Ciencia y Tecnología Agraria

Herramientas Biotecnológicas Aplicadas a la Mejora Genética de Melocotón y Nectarina

Margarita Pérez Jiménez

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CONFORMIDAD DE SOLICITUD DE AUTORIZACIÓN DE DEPÓSITO DE TESIS DOCTORAL POR EL DIRECTOR DE LA TESIS

D. José Enrique Cos Terrer, Director de la Tesis doctoral "Herramientas biotecnológicas aplicadas a la mejora genética de melocotón y nectarina"

INFORMA:

Que la referida Tesis Doctoral, ha sido realizada por D. Margarita Pérez Jiménez, dando mi conformidad para que sea presentada ante la Comisión de Doctorado, para ser autorizado su depósito.

La rama de conocimiento por la que esta tesis ha sido desarrollada es:

X Ciencias
Ciencias Sociales y Jurídicas
Ingeniería y Arquitectura

En Cartagena, a 12 de Septiembre de 2012

EL/LA DIRECTOR/A DE LA TESIS

Fdo.: José Enrique Cos Terrer

COMISIÓN DE DOCTORADO

Universidad Politécnica de Cartagena

CONFORMIDAD DE SOLICITUD DE AUTORIZACIÓN DE DEPÓSITO DE TESIS DOCTORAL POR EL/LA DIRECTOR/A DE LA TESIS

D. Antonio Asensio Calderón García, Tutor de la Tesis doctoral "Herramientas biotecnológicas aplicadas a la mejora genética de melocotón y nectarina"

INFORMA:

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POR LA COMISIÓN ACADÉMICA DEL PROGRAMA

D. Francisco Artés Hernández, Presidente/a de la Comisión Académica del Programa de doctorado en Técnicas Avanzadas en Investigación y Desarrollo Agrario y Alimentario

INFORMA:

Que la Tesis Doctoral titulada, " Herramientas biotecnológicas aplicadas a la mejora genética de melocotón y nectarina", ha sido realizada por Dª. Margarita Pérez Jiménez. bajo la dirección y supervisión del Dr. José Enrique Cos Terrer.

En reunión de la Comisión Académica de fecha 03/09/12, visto que la mencionada tesis doctoral tiene acreditados los indicios de calidad, requeridos para el depósito de tesis doctorales, regulados en el artículo 32 del Reglamento de Estudios Oficiales de Máster y Doctorado de la UPCT, y la autorización del Director de la misma, se acordó dar la conformidad para que a dicha tesis le sea autorizado, por la Comisión de Doctorado, su depósito.

La Rama de conocimiento por la que esta tesis ha sido desarrollada es:

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☐ Ingeniería y Arquitectura

En Cartagena, a 12 de Septiembre de 2012

EL PRESIDENTE DE LA COMISIÓN ACADÉMICA DEL PROGRAMA

Pdo. Francisco Artés Hernández

COMISIÓN DE DOCTORADO

UNIVERSITÀ DEGLI STUDI DI MILANO
DIPARTIMENTO DI SCIENZE AGRARIE E AMBIENTALI
PRODUZIONE, TERRITORIO, AGROENERGIA

Maria Claudia Piagnani, PhD

[redacted]

To Whom It May Concern

As the Person in charge for the in vitro culture lab of the above mentioned Department, belonging to the University of Milano (Italy), given that:

- having red the abstract and the articles belonging to Margarita Pérez Jemenéz's Doctoral dissertation
- it is only with the use of biotechnology that can be solved challenges, within a reasonable time, with fruit trees
- and finally I have been directly involved in a similar project and I have to admit it was a hard work;

considering all these mentioned above aspects the results of the experiments conducted by Margarita Pérez Jemenéz, especially those related to endogenous hormones content, representing an innovative contribution particularly in a recalcitrant species such as Peach.

Faithfully

Maria Claudia Piagnani

[redacted]

Milano, September 10th, 2012

Dipartimento di Scienze Agrarie e Ambientali - Produzione, Territorio, Agroenergia
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4 place Jussieu
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TO WHOM IT MAY CONCERN

Hereby I, Anne Guivarc'h, head of the "Mecanisms of Shoot Regeneration" team of the *Physiologie Cellulaire et Moléculaire des Plantes* (Cellular and Molecular Plant Physiology) unit, which belongs to the *Université Pierre et Marie Curie* (Pierre and Marie Curie University), located in Paris (France), having read the abstract and the articles, both published and sent, belonging to Margarita Pérez Jiménez's doctoral dissertation, certify that the carried-up researching and the obtained result are far above the minimum quality required for obtaining the PhD title. With regards to the previously mentioned results, I can also stand that they involve an important move forward as to previously-used means, this regeneration technique opening a new way which will be of great aid in future genetic transformation experiments.

Paris, July 20th, 2012

Dr Anne GUIVARC'H

[redacted]

Bât C 2 et 3 étage – Case courrier 156
4 Place Jussieu -75252 Paris cedex 05
[redacted]

A mis abuelos y a mi madre,
por esos veranos entre
melocotoneros.

«De tanto sabrosísimo bocado,
de platos que son raros
(y algunos de los cuales son muy caros),
no tengo más remedio que decir,
y vosotros lo tendréis que admitir,
que nada es comparable
al aroma en verdad inmejorable
y al sabor agradable y estimable
¡de este MELOCOTÓN tan formidable!»

Roald Dahl
(James y el melocotón gigante)

AGRADECIMIENTOS

Durante el transcurso de esta tesis ha habido una persona que ha corregido mis textos, me ha asistido como técnico de laboratorio y que además ha sido mi compañero de viaje, haciendo posible tantos momentos maravillosos fuera de casa. Andrés, gracias por ser y por estar, una parte importante de esta tesis te la debo a ti.

Gracias a mi director de tesis, Pepe, por la confianza y fe que ha depositado en mí desde el principio y durante estos cuatro años. Has demostrado una generosidad casi imposible de encontrar en ciencia. Muchas gracias por todo.

A «mi» equipo de Fruticultura, a los que están y a los que estuvieron. Paco, ese niño grande; Alfonso, siempre dispuesto a comprender y ayudar; Belén, eficiencia donde la haya, compañera y amiga, muchas gracias por todo; Marcos, las charlas en el «bus» y los sueños de futuro; Domingo que siempre tiene algo con que amenizar cada jornada; Goyo y Almudena por su compañía y su sosiego. Antonio, los datos de campo, los viajes a Cartagena y el complemento de Pepe. A Diego por hacerme ver lo que es la clase y el saber estar en una persona.

A Mercedes por las charlas, por su sabiduría y su ayuda. Gracias por estar ahí cuando te necesité, me hiciste mucha compañía, muchísimas gracias. A María mi amiga y compañera desde hace ya algún año, se que hagamos lo que hagamos siempre estaremos ahí. Mis compis de tesis Salva y Carlos, confidentes durante la montaña rusa que supone hacer la tesis.

A mi amiga y compañera de tesis Carmen, que sabe vivir como nadie y aunque sea desde la distancia siempre me contagia su entusiasmo y alegría.

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los «boys» y por supuesto Stacy, que me hizo ver que lo fácil que es vivir. A la gente del CEBAS, muchas gracias a Elena Cantero y Paco Pérez Alfocea, por acogerme durante un mes en vuestro equipo y ayudarme con las muestras.

A Marcos Egea por sus consejos y charlas, porque siempre ha estado ahí para ayudar y nunca ha puesto una mala cara sino todo lo contrario.

Antonio Calderón, una persona maravillosa siempre dispuesta a ayudar incluso más de lo que uno le pide, muchas gracias por ser mi tutor.

Y por último quisiera agradecer al antiguo Ministerio de Educación y Ciencia y al INIA que me dieran la oportunidad de hacer lo que más me gusta durante estos cuatro años, y por supuesto a Julián Barrera y su equipo, una persona eficaz, competente e impecable en su ayuda y coordinación durante este tiempo, gracias por hacer tan bien su trabajo.

Os lo debo a todos, ¡Muchas gracias!

ABSTRACT

The world population is currently growing in a global food crisis framework, in which science has to rise to the occasion. In the last decades, plant science has offered alternatives to the world, including a deep understanding and improvement of crop quality. Several techniques have been developed in order to increase production or quality by means of adapting horticulture to environmental conditions. Plant breeders have been searching for crop improvements in terms of solutions for different problems, namely diseases, production or postharvest problems. But breeders have also varied objectives such as adaptation of new cultivars to growing areas, disease resistance, ripening fruit, high productivity and organoleptic quality. However, the results obtained in classical breeding programs are limited by a methodology that has not been updated for decades: pollen collection, hybridization, crop harvest, seed germination, and transplanting to the field. Other important factors are seeds containing immature embryos. They are due to a short period between blooming and ripening that leads to low percentages of germination. Immature embryo rescue techniques provide an alternative means to recover seeds from early ripening fruit, which usually fails to completely develop in vivo. But also, fruit breeding programs are facing two limiting factors: long reproductive cycles and long juvenile phases. Thus, the combination of all of them rises the average release time of a new cultivar to a 10-year range.

Peach (*Prunus persica L.* Batsch) is one of the major fruits in the world. Thus, the economic value of this crop is one of the bases of agriculture in Europe. Currently, after decades of breeding, breeding still requires too much time obtaining peach fruits with interesting characteristics because traditional techniques are being used and the genetic basis of economically important traits are unknown. But also, in spite of the presence of some 70 peach breeding programs, peach breeding programs have failed to stabilize certain characters after many cycles of selection. This is the case of resistance to Plum Pox Virus (PPV), widely known as Sharka, a disease with high capacity for viral infection, which affects the productivity and fruit quality of *Prunus* species.

Biotechnology has been presented as a tool to overcome the limitations of classical improvement, through the incorporation of desirable genes that either confer resistance to diseases or boost the beneficial properties of some species. In this sense, genetic transformation is an important tool that can be used in different ways. This technique is very well known as an application to provide protection to crops against certain diseases or

insect actions. However, as the genome of different plant species has been sequenced, genetic transformation has played a major role in the elucidation of gene function.

To efficiently harness genetic transformation technology, an efficient plant regeneration protocol from plant cells is required. Although in some species this phenomenon occurs almost spontaneously, in others such as woody plants it is quite difficult to achieve. Peach is a particularly recalcitrant species in this sense, in which only some authors have reported somatic regeneration protocols, and in most cases from zygotic tissue. The drawback of using seed-derived material for genetic transformation techniques are that each genotype is unique and not a clone of the parent. The development of plant regeneration protocols from mature tissues is important for the modification of desirable commercial cultivars that have been selected because of economic potential. Since cultivars are typically heterozygous, the seed they produce segregate and the desirable traits may not be present in the progeny that they produce.

This research focuses in the development of protocols for both in vitro rescue of embryos and somatic regeneration in peach. On one hand, the searching for a general and improved protocol for embryo rescue in peach can provide a supplementary tool for the peach and nectarine breeding programme developed in IMIDA. On the other hand, developing a series of studies that lead to achieve an efficient protocol for plant regeneration will contribute to take a step forward in genetic transformation in peach. Preliminary field studies of genetic transformation are also included in this present dissertation.

CHAPTER I: In vitro embryo rescue

Fruit from twelve hybrid crosses performed in February 2010 was collected and the effects of the carbon type and concentration, stratification duration and gibberellic acid were tested on *in vitro* germination. Seeds were extracted from the fruit and were cultured in woody plant medium supplemented with sucrose, glucose and sorbitol at concentrations of 15, 30 and 45 g l^{-1}. The root and hypocotyl lengths were measured after the cooling process and before acclimatization. Additionally, pulses of gibberellic acid combined with different periods of stratification in the immature seeds were evaluated as well as the effects of adding gibberellic acid to the media. Germination rates and root and hypocotyl lengths were analysed.

As a result, glucose at a concentration of 15 g l^{-1} and sucrose at 30 g l^{-1}, produced higher development of the organs and growth of the seedlings. Longer stratification duration along with adding gibberellic acid to the media produced significant effects on seed germination and plant development. Pulses of gibberellic acid before germination did not induce any effect on organs length or germination. These experiments enable the establishment of a more accurate protocol for the wide range of genotypes produced in a peach breeding programme.

Several studies aimed at the achievement of a somatic regeneration protocol. Therefore, Chapters II to IV are focused on this goal. Such studies are important in order to advance in a method for genetic transformation in peach. The research approach adopted in this dissertation includes callus induction (Chapter I) and a protocol for somatic regeneration (Chapter II). As the differences in regeneration rates between genotypes were high, further studies for endogenous hormone quantification were developed (Chapter IV). This quantification was also carried out in cotyledons showing direct somatic embryogenesis.

CHAPTER II: Callus induction

We describe an efficient protocol for callus induction from adult tissues for peach. Three different commercial peach genotypes, EarlyMay®, ZiseMay® and UFO-3® were utilized, plus three other genotypes from hybrid crosses performed in February 2006: PS108, PS208, and PS708. Thirteen explant treatments were tested from nine different plant parts. Murashige and Skoog and Woody Plant Medium salts were assayed with several concentrations of 2,4-dichlorophenoxyacetic acid (2,4-D), kinetin (KN) and thidiazuron, and two different photoperiods were applied, a 16-hour photoperiod or continuous darkness. In terms of the quantitative response, two parameters were assessed: days to callus induction, and relative callus growth recorded after 30 days. Woody Plant Medium supplemented with 2,4-D and KN significantly increased the rates of callus induction in the majority of treatments. And no significant differences among the genotypes were found. The stem and calyx explants produced up to 85 and 96 % callus induction, respectively. This protocol described could be used for efficient callus induction in different *Prunus* spp.

CHAPTER III: Somatic regeneration

Somatic peach plants were regenerated from callus derived from the base of stem explants of the scion cultivars UFO-3®, Maruja®, Flariba® and Alice Bigi®, and the peach x almond rootstocks Garnem® and GF677®. A protocol for organogenic plant regeneration was developed using three culture media containing different concentrations of 6-benzyladenine (BA) and indolebutyric acid to produce organogenic calli. Shoots were obtained from sliced calli after their transfer to a differentiation culture medium containing 2 mg l^{-1} BA and 1 mg l^{-1} a-naphthalene acetic acid. Using this procedure, up to 29 regenerated plants per callus were obtained. The highest regeneration rate was obtained with the peach 9 almond rootstocks. This work provides an effective protocol that could be utilized for peach transformation research.

CHAPTER IV: Endogenous hormones analysis

PART I

Cotyledons of peach (*Prunus persica* L. Batsch cv. ZiseMay®) were cultured in vitro on medium deprived of plant growth regulators. Two different lines varying in their embryogenic (E) capacity were studied after 90 days in culture media. Endogenous levels of abscisic acid (ABA), indole-3-acetic acid (IAA), zeatin (Z), zeatin riboside (ZR), the ethylene-precursor 1-aminocyclopropane-1-carboxylic acid (ACC), salicylic acid (SA) and jasmonic acid (JA) were analysed in E and non-embryogenic (NE) cotyledons. Although no significant differences were observed in total ABA, IAA, ZR, SA and JA concentrations between the E and NE cotyledons, lower Z, ACC, Z/IAA levels were related with the E capacity of the cotyledons. These results suggest that the difference in somatic embryo formation capacity observed between E and NE cotyledons is related to their endogenous Z contents and the endogenous hormonal balance Z/IAA is an important index defining the E potential in peach cotyledons.

PART II

The relationship between endogenous hormones content and the induction of somatic peach plant was studied. To induce multiple shoots from callus derived from the base of stem explants of the scion cultivars UFO-3®, Flariba® and Alice Bigi®, and the peach x almond rootstocks Garnem® and GF677®, propagated plants were cultured on Murashige and Skoog salts augmented with 0.1 mg l^{-1} of indolebutyric acid, 1 mg l^{-1} of 6-benzylaminopurine and 3 % sucrose. The highest regeneration rate was obtained with the

peach x almond rootstocks. Endogenous levels of abscisic acid (ABA), indole-3-acetic acid (IAA), zeatin (Z), zeatin riboside (ZR), ethylene precursor 1-aminocyclopropane-1-carboxylic acid (ACC), salicylic acid (SA), and jasmonic acid (JA) were analysed in the organogenic callus. Lower levels of several hormones, namely Z, ZR, ABA, and ACC were found in the peach x almond rootstock compared to peach cultivars, while IAA and SA presented inconclusive returns. These results suggest that the difference in somatic organogenesis capacity observed in peach and peach x almond hybrids is markedly affected by the endogenous hormonal content of the studied genotypes.

Once a somatic regeneration protocol was developed, the research of this dissertation focused on the study of the different variables concerned in genetic transformation.

CHAPTER V: Genetic transformation

Different studies involved in genetic transformation were developed in peach cultivars Alice Bigi® and UFO-3® and peach x almond rootstock Garnem®. Peach callus and the base of peach shoots were infected with the strains of *Agrobacterium tumefaciens* EHA 105 and pMP90 containing the plasmid pBin19-s*gfp*. This binary vector carries the s*gfp* gene as well as the chimeric neomycin phosphotransferase and β-glucuronidase genes.

Kanamycin was used as a selectable agent. Explants sensitivity to kanamycin was tested at the concentrations of 0, 25, 50, 75 and 100 mg l^{-1}. Callus development was inhibited at the concentration of 75 mg l^{-1}. However, the tissues were highly damaged by this concentration. Therefore, this results recommended kanamycin at the concentration of 50 mg l^{-1} as selectable agent.

The addition of acetosyringone to the infection medium as well as washing the explants in a tween solution before transformation was tested regarding the number of GFP points founded in the explants. Acetosyringone effect increased, decreased or did not affect depending on the genotype and the *A. tumefaciens* strain. A decreasing of the number of GFP points was detected in explants washed in the tween solution.

Finally, explants of Garnem® were held under darkness for 60 days before the transformation event. As a result, GFP points were detected for the first time in this genotype.

These results suggest that genetic transformation in peach requires the study of different parameters in every genotype in order to set a reliable protocol for obtaining genetic modified plants.

The findings from this research provide evidence that differences between genotypes are determinant in terms of regeneration and somatic transformation. Therefore, it would be very difficult to develop a general protocol in peach, mainly for genetic transformation because marked differences were founded between genotypes; it would be advisable to study each genotype individually. However, in vitro rescue of immature embryos is a tool that will be applied to new genotypes. Hence, the main conclusion drawn from this study is that a general protocol which increases the rates of regeneration in a wide range of genotypes is mandatory.

This dissertation contributes to the advance of the biotechnological tools involved in peach and nectarine breeding. Although, further studies are recognizably necessary the studies described in this current dissertation are undoubtedly a step forward in the consecution of genetically modified peach plants.

JUSTIFICACIÓN Y OBJETIVOS

En la Región de Murcia, la fruticultura presenta una gran importancia económica, destacándose el melocotón como el frutal de hueso al que más extensión se dedica. Éste papel destacado del melocotón, propició la unión entre sector productivo e investigación, dando como resultado el programa de mejora genética IMIDA-NOVAMED de melocotón y nectarina. Desde sus inicios, este programa tuvo como objetivo la obtención de variedades mejoradas genéticamente seleccionadas en la zona de cultivo, evitando el pago de costosos royalties por parte de los agricultores y donde ellos mismos seleccionaran los objetivos de las variedades a obtener.

Sin embargo, los resultados obtenidos en programas de mejora genética clásica de melocotón y nectarina, se ven limitados por una metodología que no ha sido renovada desde hace décadas: recolección de polen, hibridación en campo, recolección de frutos, cultivo de embriones y/o germinación de semillas y traspaso de plantas a campo. Además, estos programas se enfrentan a dos factores limitantes, ciclos reproductivos largos y largos períodos de junenilidad. Así, la conjunción de todas éstas técnicas completan un ciclo, elevando el tiempo medio de registro de una variedad a 10 años.

Uno de los objetivos primordiales en un programa de mejora genética de frutales es la obtención de variedades que adelanten en su salida al mercado a sus competidoras. Para alcanzar este objetivo se realizan hibridaciones empleando variedades de rápida maduración. En la mayor parte de los casos, cuando el parental de rápida maduración es el femenino, las semillas resultantes no puedan germinar por sí solas. Así, nacieron las técnicas de cultivo *in vitro* de embriones, que suponen un excelente complemento para los programas de mejora clásica, ya que proporcionan a las semillas inmaduras de un ambiente propicio para su germinación.

Por otro lado, hay determinados caracteres que tras años de mejora no han conseguido transmitirse. Este es el caso de la resistencia a Sharka, una enfermedad viral con gran capacidad de infección cuya fuente de resistencia se encuentra en otras especies y en variedades que no están adaptadas a nuestras condiciones de cultivo. En los últimos años, la biotecnología se presenta como alternativa a este tipo de limitaciones de la mejora clásica, mediante la incorporación de genes de interés que, o bien confieren resistencia para determinadas enfermedades, o bien potencian las propiedades beneficiosas de determinadas especies. Para la consecución de estos objetivos mediante técnicas de transformación

genética se requiere de una regeneración *de novo* de plantas a partir de células vegetales individuales en cultivo *in vitro*. Sin embargo, aunque en algunas especies este fenómeno ocurre de forma casi espontánea en otras como las leñosas, es un proceso bastante dificultoso. El melocotón es una especie especialmente recalcitrante en este sentido, donde solo algunos autores han publicado protocolos de regeneración somática, y en la mayoría de los casos a partir de tejidos zigóticos. El inconveniente de la utilización de estos tejidos para técnicas de transformación genética radica en que las semillas son nuevos genotipos en los que sus cualidades organolépticas están aún por determinar.

Así, se establecieron unos objetivos que supusieran un avance biotecnológico que pudiera apoyar la mejora genética de melocotón y nectarina en la Región de Murcia. Por un lado, proceder al perfeccionamiento del protocolo de rescate *in vitro* de embriones utilizado para la germinación de semillas precoces procedentes del programa de mejora genética del IMIDA. Y por otro lado, el establecimiento de una vía de regeneración somática de melocotón y nectarina que permitiera el avance de las técnicas de transformación genética de esta especie, estancadas por la ausencia de un protocolo repetible y fiable de regeneración.

ÍNDICE

ANTECEDENTES

1 EL MELOCOTONERO

1.1 DESCRIPCIÓN TAXONÓMICA Y BOTÁNICA DE LA ESPECIE

El melocotonero (*Prunus persica* L. Batsch) pertenece a la familia de las *Rosáceas,* subfamilia *Prunoidea,* género *Prunus,* subgénero *Amygdalus,* y especie *persica.* Debe el nombre de su especie a su origen, Persia (actual Irán) de donde se extendió al resto del mundo teniendo presencia predominante en países de climas templados. A éste subgénero pertenecen especies tan populares como el almendro, y abarca tres tipos o formas diferentes: *vulgaris* o melocotón común, *laevis* o nectarina, y platicarpa o paraguayo. Es un cultivo autógamo (existen algunas variedades alógamas como J.H. Hale, Alberta, Candoka, Mikado y Alamar) y bastante compatible a la hora de realizar injertos y polinizaciones, lo que presenta una gran ventaja para la obtención de nuevas variedades. Es una especie de climas templados y su fecha de floración viene determinada por sus requerimientos de frío, variando entre 50 y 1.500 horas por debajo de 7 ºC (Bassi y Monet, 2008). El momento crítico para la obtención del fruto se sitúa en primavera, ya que los órganos florales son muy sensibles a las bajas temperaturas en estas fechas.

Figura 1. Detalles de los distintos órganos del melocotonero.

P. persica (L.) Batsch es una especie diploide (2n=16) y su nombre común es melocotonero o duraznero. Son árboles de porte medio (hasta 8 m) con hojas lanceoladas, glabras y aserradas, que alcanzan su mayor anchura aproximadamente en su punto medio y

poseen pecíolos glandulares. Las flores son solitarias y generalmente rosáceas, pero excepcionalmente pueden ser blancas o rojas. El árbol es de porte globoso y poco ramificado, de raíces ramificadas y poco profundas, corteza gris con tonalidad rojiza y hojas brillantes y alternas. El fruto es una drupa de al menos 3 cm de diámetro con pericarpo membranoso y mesocarpo carnoso; el endocarpo es leñoso, rugoso y surcado. El color del fruto puede variar, siendo blanco, amarillo, anaranjado, con o sin chapa, o rojo, al igual que el color de la pulpa; si la piel, generalmente pubescente, es glabra se determinan nectarinas. Hay dos formas predominantes, redondeada y achatada calificándose entonces de platicarpos o paraguayos y si la piel es glabra entonces se denominan platerinas.

1.2 IMPORTANCIA ECONÓMICA DEL CULTIVO

El melocotón es el fruto de hueso más importante del Mundo. En el año 2010 la producción mundial de melocotón y nectarinas fue en torno a los 20 millones de toneladas. España produjo alrededor de 1,2 millones, lo que supone casi el 6 % de la producción mundial y situándose 3ª en el ranking de mayores productores solo por detrás de China e Italia y por delante de EE. UU. (Figura 2).

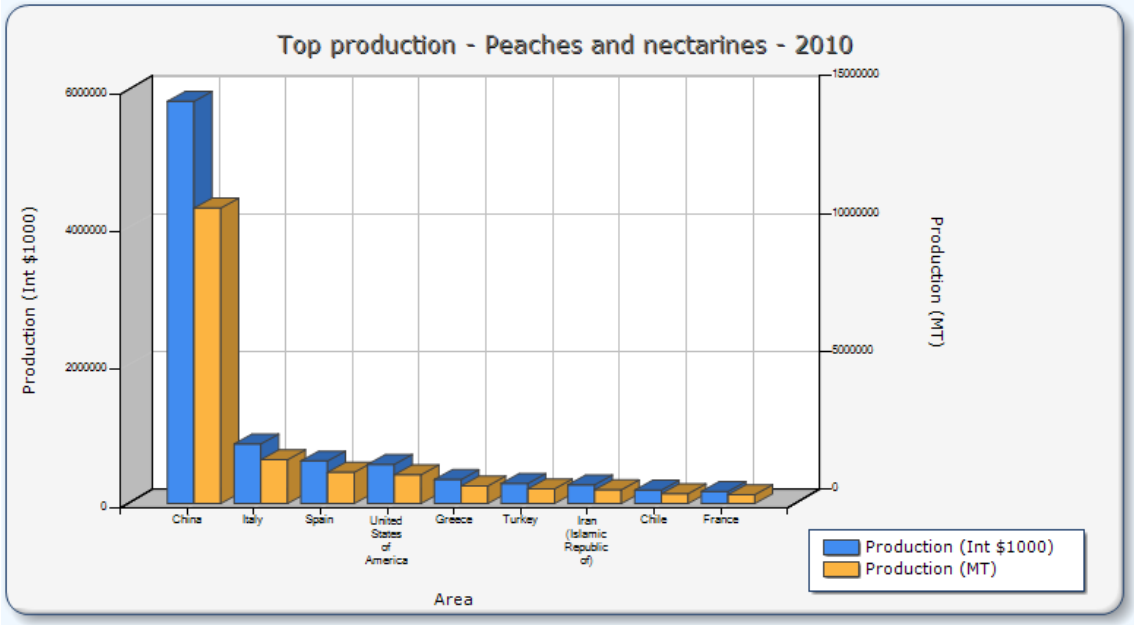


Figura 2. Producción de melocotón, en toneladas, en los principales países productores (FAOSTAT, 2010). (http://faostat.fao.org/site/339/default.aspx)

Aunque la contribución española a la producción mundial de melocotón ya es importante per se, las exportaciones son el indicador que realmente sitúan a España en una posición dominante (Figura 3). Mientras las producciones occidentales se han mantenido más o menos estables en los últimos 12 años, la producción de melocotón en China se ha incrementado fuertemente, aún así, la importancia de China como primer productor es limitada, ya que la mayor parte de su producción queda para consumo interno siendo las exportaciones casi nulas. En cambio España solo consume una pequeña parte del melocotón que produce, enviando el resto principalmente al mercado europeo.

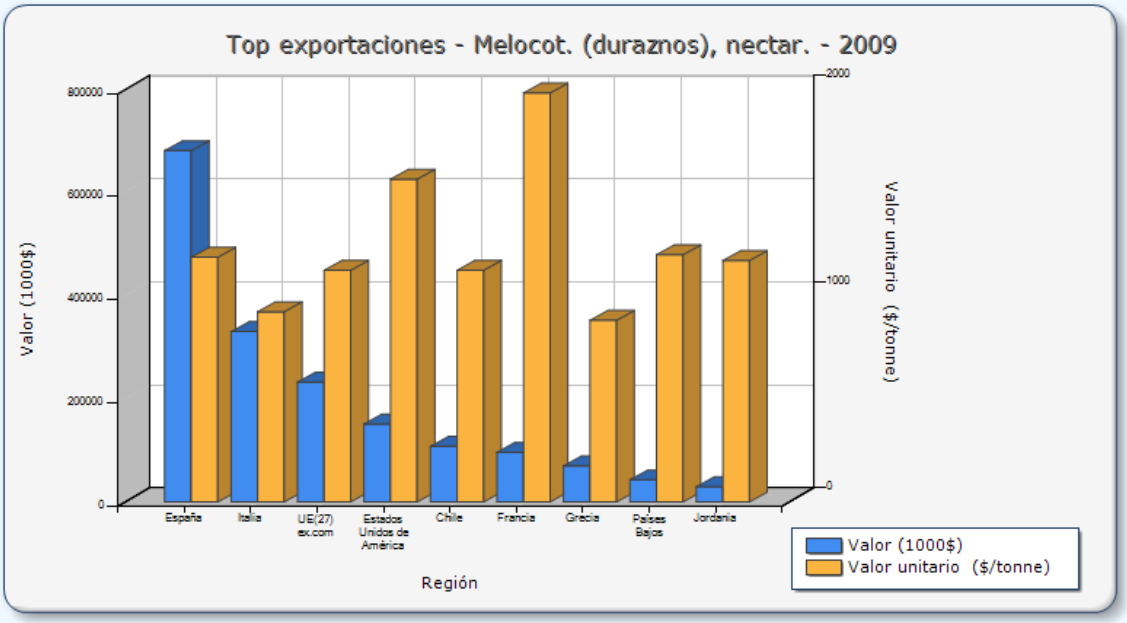


Figura 3. Principales países exportadores de melocotón y nectarina expresados en toneladas exportadas (FAOSTAT, 2009). (http://faostat.fao.org/site/339/default.aspx)

El melocotón es el frutal de hueso más importante, no solo en España, sino también de la Región de Murcia. La superficie dedicada en Murcia al cultivo del melocotón se incrementa año a año, siendo en el año 2011 de 17.519 ha, lo que constituye casi el 22,5 % de la superficie total dedicada al melocotón en toda España (Tabla 1). En términos de producción la Región de Murcia produjo 121.147 t en el año 2009, lo que implica un aporte del 20 % de la producción nacional, solo por detrás de Aragón y Cataluña (Tabla 2).

Tabla 1. Superficie cultivada de melocotón y nectarina en España por Comunidades Autónomas en el año 2011. (http://www.marm.es/es/agricultura/estadisticas/)

Comunidades Autónomas	Superficie (ha)
Cataluña	20.891
Aragón	18.399
R. de Murcia	17.519
Andalucía	7.208
Extremadura	6.028
C. Valenciana	5.607
La Rioja	746
Galicia	601
Castilla la Mancha	450
Navarra	369
Canarias	62
Castilla y León	29
P. De Asturias	8
Baleares	1
Cantabria	0
Madrid	0
País Vasco	0
ESPAÑA	77.917

Tabla 2. Producción de melocotón y nectarina en España por Comunidades Autónomas en el año 2009. (http://www.marm.es/es/agricultura/estadisticas/)

Comunidades Autónomas	Producción melocotón (t)	Producción nectarina (t)	Total (t)
Cataluña	189027	118823	307.850
Aragón	202.795	100.302	303.097
R. de Murcia	199.518	46.315	245.833
Andalucía	71.235	81809	153.044
Extremadura	57.777	63.370	121.147
C. Valenciana	20.504	23.318	43.822
Navarra	18.280	2.002	20.282
La Rioja	16.267	2.432	18.699
Galicia	11.449	0	11.449
Castilla la Mancha	6.181	340	6.521
Canarias	2.020	0	2.020
Castilla y León	447	0	447
P. de Asturias	300	0	300
Baleares	201	30	231
País Vasco	130	0	130
Madrid	14	0	14
Cantabria	0	0	0
ESPAÑA	796.145	438.741	1.234.886

1.3 MEJORA GENÉTICA DE MELOCOTÓN Y NECTARINA

Los árboles frutales presentan una baja incidencia de mutaciones espontáneas de interés agronómico. Estos, se caracterizan por tener etapas de juvenilidad y ciclos reproductivos largos, que hacen de los programas de mejora, proyectos a largo plazo. Por ello, los estudios genéticos intensivos constituyen la base para un buen programa de mejora de melocotón y nectarina, donde los resultados se ajusten a los objetivos y los plazos puedan acortarse. Actualmente existe unos 70 programas de mejora genética en todo el mundo intentando obtener variedades adaptadas a sus condiciones de cultivo, ya que las diferencias edafoclimáticas de las distintas áreas de producción ejerce una presión ambiental en las plantas variando en muchas ocasiones sus fenotipos. Inicialmente los programas de mejora se llevaron a cabo en instituciones públicas como universidades e institutos de investigación, aunque en la actualidad existen una gran cantidad de mejoradores privados como Zaiger o Bradford (California - EE. UU.), Euro-Pepiniers, Minguzi, Nicotra, Bellini, Fideguelli, Maillard-ASF, PSB-Buffat, Agromillora, Provedo-Caval, Agroselection Fruit o Escande que han invertido sus esfuerzos al ver la oportunidad de negocio existente (Byrne, 2002). El uso de las variedades surgidas de estos programas de mejora por los agricultores estaba supeditado al pago de royalties a sus obtentores. Hace más de una década los productores evitaban el canon propagando variedades registradas ilegalmente y cambiándole el nombre a las variedades que cultivaban. Con el perfeccionamiento de técnicas moleculares de detección de huella genética, y su uso por parte de las agrupaciones de defensa de variedades registradas, esta práctica a entrado en desuso impulsando el desarrollo de nuevos programas de mejora donde el agricultor tiene derechos legales sobre las variedades que cultiva.

Dentro de la especie *P. persica* hay una gran diversidad varietal. Desde 1980 se han registrado más de 1.700 variedades de melocotón en el mundo (Della Strada y Fideghelli, 2008) y para caracterizarlas se describen diferentes caracteres del árbol, brote, flores, hoja y fruto mediante los descriptores sugeridos por Zielinski (1955), Bellini y Scaramuzzi (1976) y Bellini *et al.* (2007). Desde los años 60 el número de nuevas variedades registradas no ha dejado de crecer, apareciendo 202 entre 1970-1980, 625 entre 1981-1990 y 1.092 entre 1991-2001, de las cuales un 56 % son de melocotón, 36 % de nectarina y 8 % de pavía (Della Strada y Fideghelli, 2008). Si se analiza el total de obtenciones presentadas en el mundo en los últimos 25 años, periodo 1991-2006, (Figura 4) se puede observar que ha

sido de 1.597, destacando EE. UU. como el principal obtentor. En Europa, Italia y Francia son los países que han obtenido un mayor número de nuevas obtenciones, situándose España en un 5º lugar con 82.

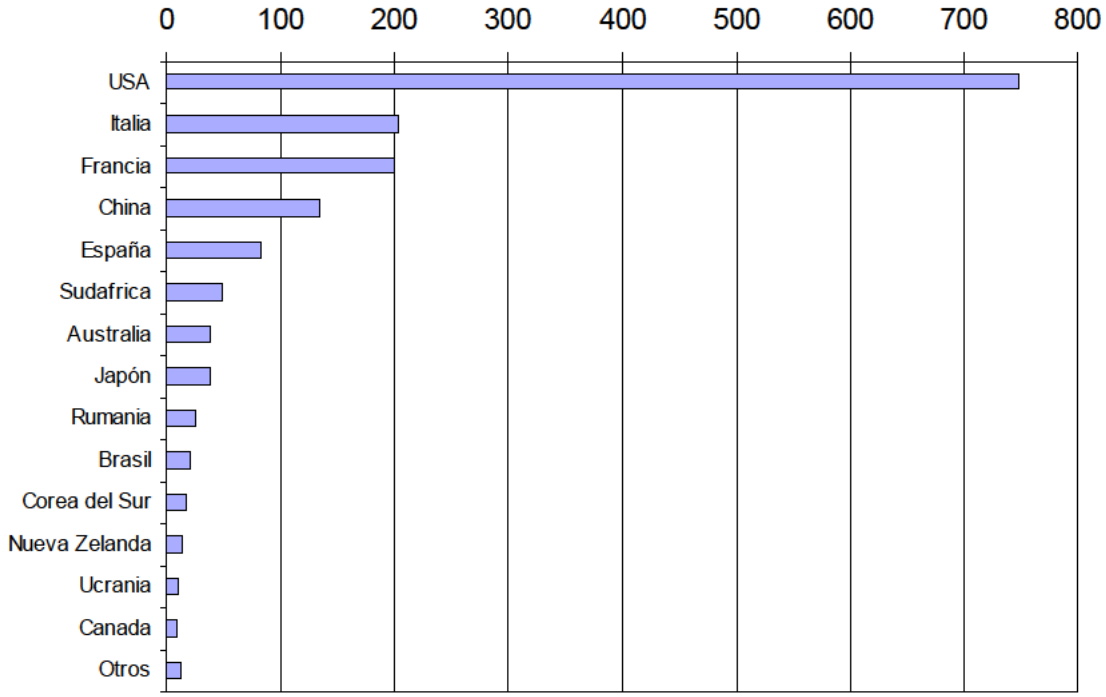


Figura 4. Número de nuevas variedades de melocotón y nectarina obtenidas en los distintos programas de mejora genética desarrollados en el mundo durante el periodo 1991-2006 (Fuente: CRA-ISF Roma, DellaStrada e Fideghelli).

Hasta ahora la mejora clásica ha producido variedades comerciales de casi todas las especies leñosas, pero tiene el inconveniente de que para la obtención de una nueva variedad pueden pasar muchos años, debido no solo a la longitud de sus ciclos reproductivos, sino también a la duración de las etapas juveniles (Tiang *et al.*, 2009). Si a esto le sumamos las labores de recolección del polen de los parentales seleccionados, la emasculación manual de las flores, la polinización, la recolección de las semillas de los frutos, el rescate de embriones, la estratificación y germinación de las semillas, el cultivo en invernadero de la progenie, su plantación en campo y las posteriores evaluaciones y selecciones, el tiempo mínimo necesario desde que se planta la nueva semilla hasta que se patenta una nueva variedad de melocotón es de 10 años, aunque en algunos casos es muy superior.

En las dos últimas décadas se han desarrollado técnicas biotecnológicas como la transformación genética que son capaces de obtener variedades mejoradas en determinados caracteres de interés (Gao *et al.*, 2010), como pueden ser resistencia a enfermedades, plagas, mayor contenido en compuestos como antioxidantes o vitaminas, y todo esto en un plazo relativamente corto. Gracias a estas técnicas podemos mejorar variedades que ya gocen de alto interés agronómico y comercial, y conferirles nuevas características que las hagan aún más interesantes para el consumidor y/o el agricultor. Las ventajas que aportan estas técnicas son además de la reducción del tiempo de obtención, que ya no existe la necesidad de establecer un carácter por medio de cruzamientos.

En el caso del melocotón las principales líneas de mejora están enfocadas no solo a unas cualidades organolépticas comerciales sino también a la resistencia a enfermedades y plagas, un porte del árbol ideal para la recolección, mayor producción sin perder calibre, homogeneidad en la maduración y tolerancia a condiciones adversas como pueden ser las heladas. Si se obtienen plantas resistentes a insectos y enfermedades se obtendrían a la vez plantas libres de plaguicidas y tratamientos. Se podría modificar el periodo de floración y el control de la maduración permitiendo al agricultor obtener cosechas fuera de las temporadas de mayor saturación de los mercados y alargar la presencia de una determinada fruta en el mercado. En lo que se refiere a la tolerancia a climas adversos se podría extender el cultivo de determinadas especies a latitudes donde hace unos años era impensable.

2 RESCATE *IN VITRO* DE EMBRIONES

Según Kester y Hesse (1995), el cultivo de embriones es una técnica mediante la cual un embrión inmaduro es aislado de la semilla y se cultiva en condiciones asépticas. El primero en utilizar este tipo de técnicas en frutales de hueso fue Tukey en 1933. En programas de mejora genética, los cruces dirigidos usando variedades precoces como parentales femeninos, provocan que ciertos embriones no sean capaces de germinar, debido a que la fruta madura antes que el embrión termine su desarrollo. Generalmente, en condiciones naturales el embrión abortaría, pero es posible recolectar la fruta antes de que termine de madurar y «rescatar» el embrión en el laboratorio en un medio de cultivo artificial que le permita germinar y desarrollarse adecuadamente. Además esta técnica presenta otra ventaja, y es que las plantas adultas germinadas en cultivo *in vitro* son más

vigorosas que las obtenidas por métodos de germinación tradicionales (Chaparro y Sherman, 1994). El éxito de la técnica depende directamente del tamaño del embrión ya que cuanto menor es el tamaño de un embrión mayor son sus requerimientos nutricionales (Ramming, 1985) y más difícil será su germinación.

Figura 5. Embrión inmaduro germinando *in vitro*.

Básicamente hay dos formas de realizar el rescate de embriones, una es separando el embrión y cultivándolo en el medio de cultivo, y la otra es hacer un cultivo de óvulos, lo que a priori es ventajoso para el embrión ya que se encuentra en un entorno materno para su desarrollo (Rubio *et al.*, 1997).

Una vez *in vitro* es especialmente importante la etapa de estratificación, donde se observa que con esta técnica el embrión aumenta su tamaño, aspecto que se expresa tanto en aumento de peso como de longitud. Es importante que los embriones pasen suficiente tiempo en frío para conseguir el mayor éxito en porcentajes de germinación, además la falta de frío hace que el porcentaje de plantas con crecimiento en roseta sea mayor (Topp *et al.*, 2008).

2.1 IMPORTANCIA DEL RESCATE *IN VITRO*

Las variedades de maduración temprana son básicamente las que más problemas presentan a la hora de germinar sus semillas, esto es debido al corto periodo entre floración y maduración. Pero, a su vez, en zonas cálidas, estas variedades son las que mayor interés tienen para el productor, ya que la variedad que primero aparece en el mercado,

normalmente es la variedad que mayor precio alcanza. Estas variedades, debido a su rápida maduración es difícil que alcancen un desarrollo aceptable del embrión para la germinación, lo que lleva a la necesidad imperiosa de un protocolo de rescate de embriones eficiente. Además, éste protocolo debe ser independiente del genotipo, ya que los nuevos genotipos obtenidos por cruces dirigidos en los programas de mejora son desconocidos para el mejorador. Sin estas técnicas las variedades de maduración temprana no podrían ser utilizadas como parentales, ya que el porcentaje de germinación sería mínimo. La época de maduración es un carácter cuantitativo, por lo que si el objetivo es obtener variedades precoces, solo usando variedades de maduración temprana como parentales, es posible obtenerlas.

3 REGENERACIÓN

Se entiende por regeneración el proceso de crecimiento de un órgano o planta a partir de una sola célula somática o grupo de ellas. Siendo la regeneración el paso previo indispensable para establecer un protocolo de transformación (Miguel y Oliveira, 1999). Así, solo induciendo el desarrollo de plantas desde tejidos somáticos se conseguiría la obtención de plantas transgénicas a partir de las células transformadas, resultando un individuo idéntico al de partida con la inclusión del gen de interés introducido en el proceso de transformación.

El desarrollo de la biotecnología vegetal ha permitido entender y controlar, al menos en parte, los factores que influyen en la morfogénesis de un tejido vegetal previamente desdiferenciado hasta obtener una planta completa (Skoog y Miller, 1957; Reinert, 1959; Steward *et al*., 1958). Dicha reorganización puede transcurrir siguiendo dos rutas alternativas, organogénesis (diferenciación de meristemos caulinares y radiculares originando tallos y raíces adventicias) o embriogénesis (formación de embriones somáticos sin un hecho previo de fecundación).

Históricamente las especies leñosas siempre han sido difíciles de regenerar *in vitro*, pero en los últimos años han ido publicándose distintos protocolos de regeneración para distintas especies, como en cerezo (Tang *et al*., 2002), pistacho (Tilkat *et al*., 2009), ciruelo europeo (Scorza *et al*., 1995a), zarzamora (Gupta el al, 2008), albaricoque (Petri *et al*., 2005) o melocotón (Hammerschlag *et al*., 1985). Aunque el melocotón es una de las especies más recalcitrantes para regeneración *in vitro* (Bhansali *et al*., 1990; Padilla *et al*., 2006), algunos

autores han asegurado haber alcanzado este objetivo. La mayoría de estos logros se han conseguido a partir de material inmaduro de semilla (Bhansali *et al*., 1990; Guohua and Yu, 2002; Scorza *et al*., 1990; Hammerschlag *et al*., 1985; Mante *et al*., 1989; Pooler y Scorza, 1995) lo que implica regenerar un material que es desconocido en genotipo y fenotipo. Gentile *et al.* en 2002, establecieron un protocolo de regeneración a partir de material adulto en su laboratorio que hasta la fecha ha sido difícil de reproducir. Ya que uno de los principales motivos de la obtención de un protocolo de regeneración es obtener variedades comerciales mejoradas genéticamente, lo que se necesitaría sería el desarrollo de un sistema de regeneración fiable y basado en tejidos adultos (Litz y Gray, 1992; Liu y Pijut 2008, 2010), donde sería particularmente útil la obtención de variedades resistentes a efectos bióticos y abióticos (Srinivasan *et al*., 2004).

En los tejidos vegetales es posible el proceso conocido como desdiferenciación, en el cual células vegetales diferenciadas en tejidos, incluso en estado maduro, guardan la capacidad para revertir su función y volver al estado meristemático (George, 1993b). Así aparece el fenómeno de totipotencia celular, donde un célula vegetal es capaz una vez desdiferenciada de volver a diferenciarse y dar lugar a una planta completa. Teóricamente cualquier célula vegetal conserva la capacidad morfogenética de producir una planta completa por sí misma, respondiendo a ciertos estímulos ambientales o genéticos. Aunque algunas especies pueden regenerar *in vitro* a partir de tejidos somáticos con protocolos sencillos, otras especies presentan más dificultad para ello. Las dificultades para regenerar ciertos genotipos podrían ser debidas a que la adquisición de competencia, inducción y diferenciación celular estarían mediadas de diferente manera en cada genotipo (Litz y Gray, 1992). La neoformación de brotes o embriones en tejido somático se denomina organogénesis somática o adventicia en el caso del desarrollo de plantas y embriogénesis somática o adventicia cuando lo desarrollado son embriones. Si este fenómeno se produce pasando por una etapa de formación de callo se dice que es indirecta y si ocurre sin pasar por esta etapa directa (Figura 6).

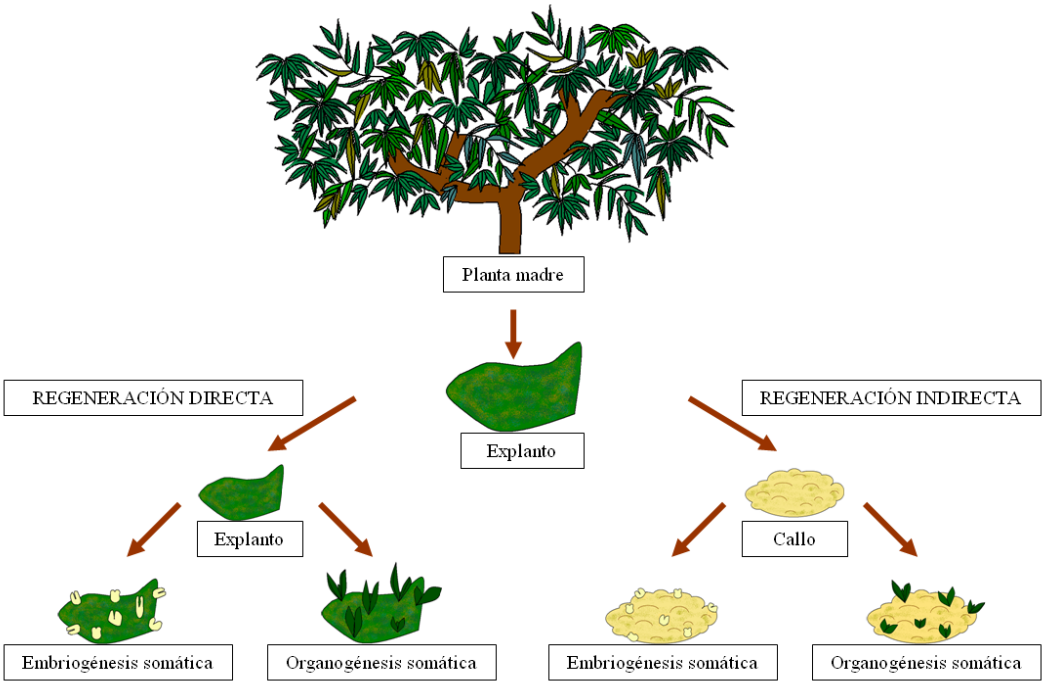


Figura 6. Etapas de la regeneración somática.

3.1 REGENERACIÓN DIRECTA E INDIRECTA

La organogénesis somática puede ocurrir directamente desde las células del explanto. Esta forma de regeneración ha sido observada raramente o es desconocida en algunas especies, y en los casos en los que se ha descrito, las evidencias histológicas han sido en algunas ocasiones insuficientes para confirmar estas observaciones (Litz y Gray, 1992). En algunas especies el hecho de producir brotes emergentes directamente de los tejidos de los explantos les confiere a estas especies un método eficaz de micropropagación. Sin embargo, la inducción o regeneración directa de brotes depende de la naturaleza del órgano de la planta que se esté utilizando y es directamente dependiente del genotipo (George, 1993a). Esta formación directa de brotes está algunas veces acompañada por la proliferación de células desorganizadas y un tejido regenerativo que podría ser clasificado como un callo (George, 1993a). Esto, ocurre normalmente cuando hablamos de tejidos vegetativos, pero se han encontrado casos de embriogénesis directa en tejidos embrionarios sin encontrar éste fenómeno.

La regeneración indirecta ocurre después de una desdiferenciación de las células, y la multiplicación y agregación de las mismas en una estructura desorganizada denominada callo. Hay muchos factores que afectan a la inducción de callo, como la naturaleza

del explanto, las condiciones físicas de luz, fotoperiodo y temperatura en las que se desarrolla, la salinidad y el pH del medio, el agente gelificante o los reguladores del crecimiento.

Las células del callo, por medio de un estímulo ambiental o genético, normalmente reguladores del crecimiento, pueden evolucionar y diferenciarse en órganos, dando lugar a brotes o embriones. La regeneración indirecta es el método más común de regeneración somática, pero conlleva el riesgo de que las plantas regeneradas difieran genéticamente de la planta madre, lo que se denomina variación somaclonal. Debido a esto, el uso de callos para su posterior propagación no es recomendado. La variación somaclonal es más frecuente en plantas regeneradas por organogénesis indirecta que las formadas por embriogénesis indirecta, aunque hay una fuerte influencia del genotipo y del origen meristemático o no del callo (George, 1993b).

3.2 ORGANOGÉNESIS Y EMBRIOGÉNESIS

La organogénesis es un fenómeno en el cual se originan estructuras monopolares de origen multicelular que establecen conexión vascular con el tejido del que derivan. Normalmente la formación de tallos y raíces ocurre de modo independiente, siendo común la ausencia de conexión vascular entre ambas estructuras (Segura, 1994). La formación *in vitro* de hojas adventicias denota la presencia de meristemos, algunas veces las hojas aparecen sin la presencia explícita de estos, en este sentido aparecen opiniones en dos sentidos sobre si estas hojas aparecen *de novo,* o si primeramente apareció un brote pero falló en su desarrollo (George, 1993a).

La embriogénesis somática es un fenómeno que se basa en la producción de estructuras embrionarias a partir de células del soma de la planta. Los embriones somáticos son estructuras bipolares que presentan tanto ápice caulinar como radicular, que han surgido a partir de una sola célula (Faure *et al.*, 1996). Los tejidos de tallo y raíz se conectan por un sólido precambium que da origen al tejido vascular, además el apéndice radicular es cerrado, sin conexión vascular con el tejido a partir del cual se ha diferenciado el embrión somático. Los embriones somáticos presentan un desarrollo igual a los embriones zigóticos y son capaces de germinar para dar plantas completas.

Según Komamine *et al.* (1992), el proceso de inducción de embriones somáticos se divide en cuatro fases: la fase 0, donde células individuales competentes forman agregados proembriogénicos en presencia de auxina; la fase 1, cuando los agregados celulares son transferidos a un medio sin auxina y se produce la activación de su división y diferenciación; la fase 2, donde se da la diferenciación de embriones en estado globular; y la fase 3, en la cual el desarrollo de embriones globulares hasta plántulas pasando por los estados de corazón, torpedo y cotiledonario (Figura 7).

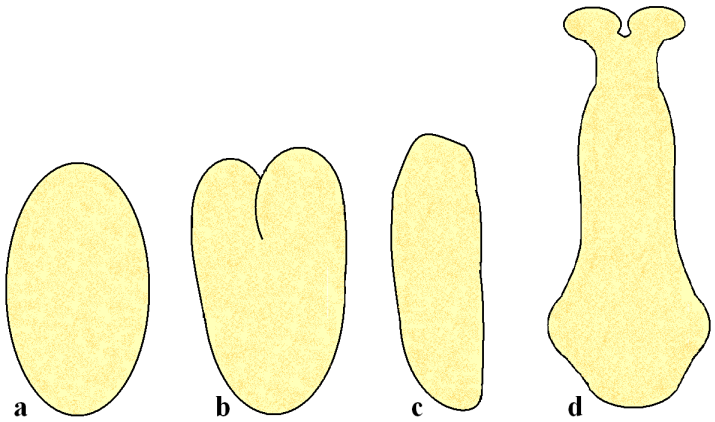


Figura 7. Estados embrionarios. **(a)** preembrión. **(b)** corazón. **(c)** torpedo. **(d)**. cotiledonario.

3.3 FACTORES QUE AFECTAN A LA REGENERACIÓN

La expresión *in vitro* de una determinada respuesta morfogenética, ya sea organogénesis o embriogénesis somática, viene determinada por la interacción y efecto de numerosos factores, los más importantes son el genotipo de la planta, el tipo de explanto y su estado fisiológico, la composición del medio de cultivo y los factores físicos del cultivo.

3.3.1 Reguladores del crecimiento

Los reguladores del crecimiento juegan un papel fundamental en el control de la morfogénesis. Pero hay una duda subyacente y es si los reguladores del crecimiento realmente condicionan la ruta morfogenética que inician las células o si por el contrario las células ya están predeterminadas a seguir esa ruta y son los reguladores del crecimiento los que activan la expresión de la misma. En cualquier caso, la hipótesis más aceptada para explicar el modo de acción de los reguladores del crecimiento en la organogénesis y la embriogénesis, postula que dicho proceso está regulado por cambios en los niveles hormonales endógenos de auxinas y citoquininas. De acuerdo con lo que se ha

denominado «efecto Skoog-Miller», la diferenciación de yemas vegetativas (organogénesis) es promovida por balances auxina/citoquinina favorables a las citoquininas, mientras que los balances favorables a las auxinas inducen la formación de raíces (rizogénesis) y/o embriones (embriogénesis). Pero no todos las auxinas y citoquininas producen el mismo efecto, ya que el tipo de citoquinina o auxina utilizada como sus concentraciones producen modificaciones significativas en los resultados (Mante *et al*., 1989). En melocotón las auxinas que mejores resultados han obtenido en regeneración han sido el ácido naftalenacético (ANA) (Hammerschlag *et al*., 1985; Svircev *et al*., 1993; Gentile *et al*., 2002), ácido indolbutírico (IBA) (Pooler y Scorza, 1995) y como citoquininas la benciladenina (BA) (Bhansali *et al*., 1990; Hammerschlag *et al*., 1985; Gentile *et al*., 2002) y el tidiazurón (Pooler y Scorza, 1995; Declerck y Korban, 1996).

3.3.2 El genotipo

El genotipo es el factor que más fuertemente influencia la morfogénesis. Aunque los balances de auxina/citoquinina permiten la regeneración somática en una gran cantidad de especies vegetales, existen muchos casos en los que no ha sido posible inducir respuestas morfogenéticas suplementando el medio con estos reguladores del crecimiento. La efectividad del balance auxina/citoquinina depende de la especie y de la variedad.

3.3.3 El tipo de explanto

El explanto también ejerce un efecto importante en el desarrollo posterior del cultivo, así como en su capacidad morfogenética. A escala bioquímica, se ha observado que el patrón de isoenzimas y el perfil de actividades enzimáticas específicas pueden ser diferentes en cultivos de callos derivados de distintos tejidos de la misma planta (López-Pérez, 2006). Parece, por ello, que las células de explantos distintos no se «desdiferencian» de la misma forma.

El éxito en regeneración de plantas en tejidos de melocotón es escaso a pesar de usar explantos juveniles como material de partida. Las semillas inmaduras se han utilizado con mayor frecuencia como explanto en melocotón (Meng y Zhou ,1981; Hammerschlag *et al*., 1985; Scorza *et al*., 1990; Bhansali *et al*., 1990; Smigocki y Hammerschlag, 1991; Svircev *et al*., 1993; Pérez-Clemente *et al*., 2004). En cuanto a tejidos no juveniles se ha obtenido un

escaso éxito, únicamente a partir de hojas (Gentile *et al*., 2002) y bases de explantos caulinares (Pérez-Jiménez *et al*., 2012).

Cada vez existen más evidencias de que no todos los tejidos vegetales cultivados *in vitro* poseen la teórica totipotencia atribuida a las plantas. Por el contrario, en ocasiones recuerdan de qué parte de la planta proceden y este recuerdo influye en la morfogénesis y en la estabilidad genética del cultivo (Lindsey y Jones, 1989).

3.3.4 Composición del medio de cultivo

Generalmente como medio de regeneración y proliferación se suele emplear el mismo medio de cultivo, a igual o menor concentración (Bassi y Cossio, 1991). En frutales, y en especial en el melocotón hay dos formulaciones de nutrientes que se repiten en la bibliografía y son el medio Murashige y Skoog (MS) (Murashige y Skoog, 1962) y el medio woody plant medium (WPM) (Lloyd y McCown, 1981) a distintas concentraciones de los mismos. La fuente de carbono más usada en especies leñosas es la sacarosa, aunque algunos estudios han demostrado que la naturaleza de la fuente de carbono usada puede tener más efecto en la regeneración del previamente estimado (Declerck y Korban, 1996). Al igual pasa con el gelificante, el uso de medios sólidos o líquidos determinan en muchas especies su comportamiento *in vitro* hasta el punto de inhibir o activar ciertos procesos como la inducción de raíces o la propagación.

Otros componentes que se pueden añadir al medio son vitaminas, carbón activo, antioxidantes, aminoácidos, captadores de etileno, extractos de levaduras y pulpas y extractos vegetales entre otros.

3.3.5 Factores físicos

Aunque de forma generalizada las condiciones de luz y temperatura suelen ser las mismas para un genotipo sin importar el tipo de cultivo *in vitro* que se está realizando, hay ensayos que indican que es aconsejable usar intensidades de luz menores a las habituales (Druart, 1990). Normalmente, los cultivos se mantienen en oscuridad durante la etapa de inducción y expresión de órganos en los protocolos de embriogénesis y en luz en los de organogénesis, aunque algunos estudios indican que no existe una dependencia directa de estos procesos con la luz o el fotoperiodo (Ning y Bao, 2007).

Hay ocasiones en las que la aplicación de campos magnéticos, eléctricos o sonicación puede estimular la regeneración, ya que produce cambios en los canales de las células que hacen que se abran y permitan el paso de sustancias que en condiciones normales no pasaría al interior celular y/o tisular.

4 HORMONAS

Algunos de los compuestos que viajan por el interior de los tejidos de la planta tienen una función reguladora más que nutritiva, estas sustancias suelen actuar en pequeñas cantidades. Se les conoce como hormonas vegetales o fitohormonas y actúan sobre diferentes procesos fisiológicos de la planta, principalmente a nivel de crecimiento, desarrollo y diferenciación. Algunas sustancias químicas sintéticas tienen la misma función que estas naturales ya que tienen estructuras químicas similares, al conjunto de todas estas sustancias, sean naturales o sintéticas se las conoce como reguladores del crecimiento.

En ocasiones los reguladores del crecimiento aportados externamente a las plantas producen variaciones endógenas de estas u otras sustancias, provocando cambios en el desarrollo de la planta (Magyar-Tábori *et al*., 2010). Con un conocimiento exhaustivo de las funciones e interacciones de hormonas endógenas y reguladores exógenos se pueden producir modificaciones en el crecimiento y el desarrollo vegetal.

Inicialmente, la cuantificación de las hormonas vegetales requería la purificación de decenas o incluso centenares de kilogramos de tejido. Sin embargo, las técnicas actuales (cromatografía gaseosa o líquida acoplada a espectrometría de masas) permiten su determinación a partir de unos pocos miligramos de material vegetal, haciendo posible el análisis individual de hojas, brotes o incluso de diferentes tejidos dentro de un órgano.

4.1 AUXINAS

Un compuesto es llamado auxina si es capaz de controlar distintos procesos como el crecimiento y la elongación celular. Las auxinas son capaces de iniciar la división celular y están implicadas en la formación de meristemos o de órganos. En un tejido organizado, las auxinas son las responsables de mantener la dominancia apical.

El uso de auxinas es muy habitual en trabajos de micropropagación, para inducir el crecimiento del callo, suspensiones celulares o de órganos y como inductores de

morfogénesis normalmente combinados con citoquininas. La elección del regulador y su concentración viene determinada por el tipo de crecimiento o desarrollo que queramos promover, los niveles endógenos de auxina en el explanto, la capacidad de los tejidos de producir auxina y la interacción de esta aplicada con otros compuestos endógenos.

La auxina natural más frecuentemente encontrada en la mayoría de las plantas es el ácido indolacético (AIA) (Figura 8) (George, 1993a). Existen diferentes precursores del AIA que a veces también presentan actividad auxínica (indolacetaldehido, ácido idol-3-pirúvico, ácido indol-3-propiónico, etc.). El AIA se sintetiza a partir del triptófano o el indol, principalmente en primordios de hoja y hojas jóvenes, así como en semillas en desarrollo. Se transporta célula a célula a través del cambium vascular y las células epidérmicas. Además, en el transporte hacia la raíz también está implicado el floema. El AIA está relacionado con cada aspecto del crecimiento y el desarrollo de la planta como también en labores defensivas. Esta diversidad funcional queda reflejada por la extraordinaria complejidad de sus rutas de biosíntesis, transporte y transducción de señal. Esta complejidad hace que los niveles de auxina estén altamente regulados ocupando un papel central en el crecimiento vegetal (Santner *et al.*, 2009).

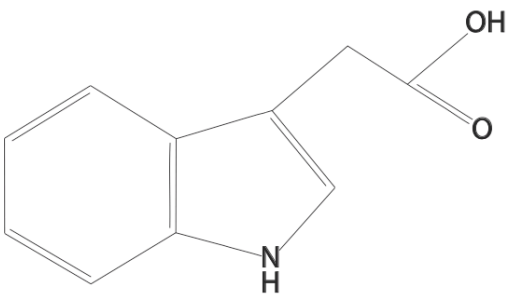
AIA	EFECTOS DE LAS AUXINAS
	• Elongación celular • División celular • Diferenciación de tejidos vasculares • Iniciación radical • Gravitropismo y fototropismo • Dominancia apical • Retraso de la senescencia • Abscisión de frutos y hojas • Crecimiento del fruto • Distribución de fotoasimilados • Retraso de la maduración de frutos • Desarrollo floral

Figura 8. Efectos de las auxinas sobre distintos procesos de la fisiología de la planta y estructura química del ácido indol-3-acético.

4.2 CITOQUININAS

Las citoquininas son sustancias derivadas de adenina. La forma química más común en plantas es la zeatina (Z) (Figura 9) aunque también pueden aparecer como ribósidos o ribótidos (Figura 10). Se sintetizan a partir de una modificación bioquímica de la adenina en

zonas apicales de la raíz y semillas en desarrollo y se transportan a través de la sabia xilemática desde la raíz hacia la parte aérea.

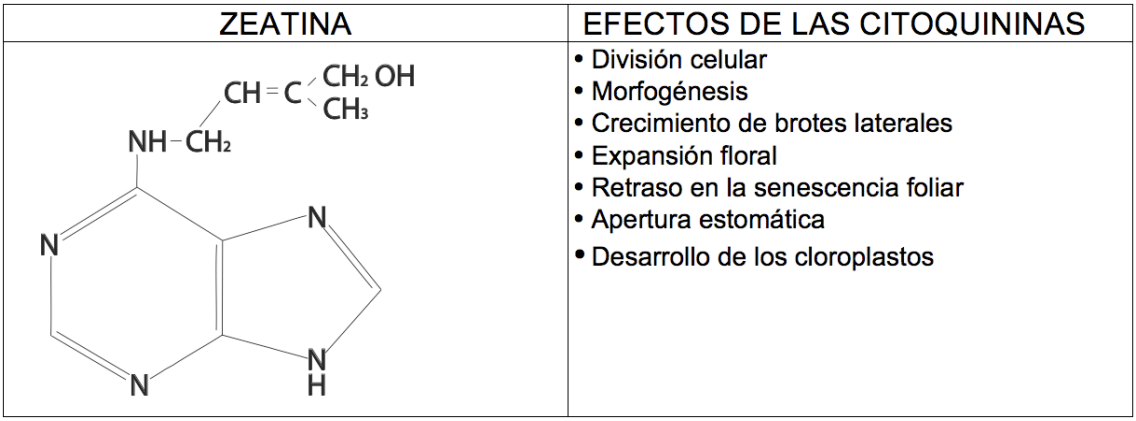


Figura 9. Principales funciones de las citoquininas en plantas y estructura molecular de la zeatina.

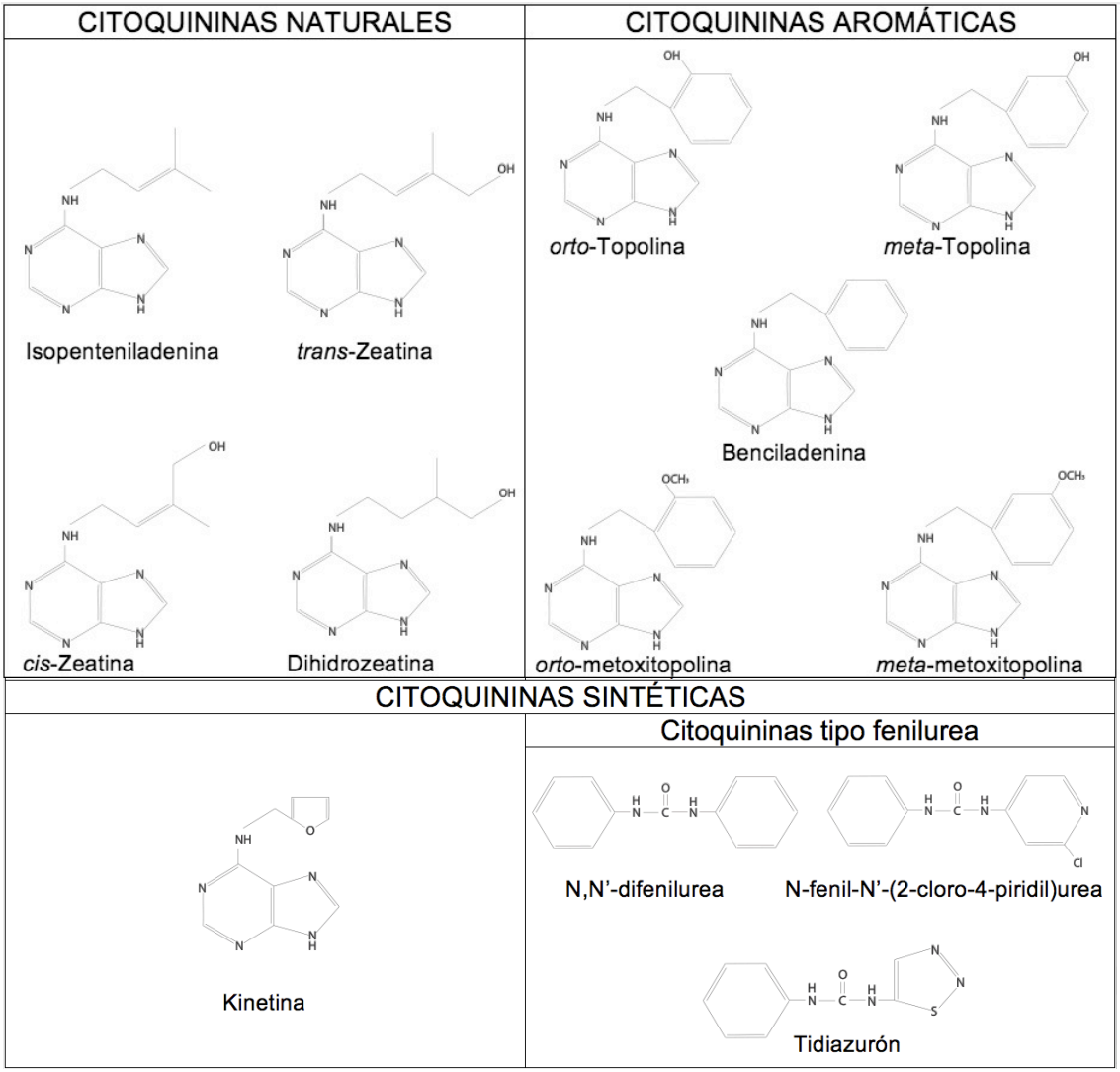


Figura 10. Estructura química de las principales citoquininas.

4.3 GIBERELINAS

Las giberelinas conforman una familia de compuestos basados todos ellos en la estructura básica *ent*-giberelano. Existen alrededor de 125 compuestos dentro de este grupo de hormonas, siendo el ácido giberélico (GA_3) un compuesto ampliamente utilizado en aplicaciones exógenas de giberelinas. Sin embargo, la giberelina más importante en plantas es GA_1 (Figura 11). Estos compuestos se sintetizan a partir del gliceraldehido-3-fosfato a través del isopentenil difosfato, en la parte aérea de tejidos jóvenes y semillas en desarrollo. Su biosíntesis comienza en los cloroplastos. Algunas giberelinas se transportan probablemente a través del floema y del xilema. Sin embargo, el transporte de GA_1 parece ser muy restringido.

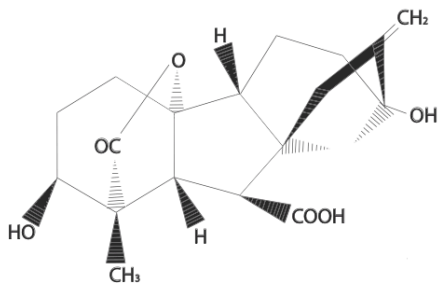
GIBERELINA A_1	EFECTOS DE LAS GIBERELINAS
	• Crecimiento del tallo (elongación de entrenudos) • Espigado en las plantas de día largo • Inducción en la germinación de semillas • Producción enzimática durante la germinación • Formación y desarrollo del fruto

Figura 11. Efectos de las giberelinas en la planta y estructura química de la giberelina A_1.

Las giberelinas constituyen un grupo de reguladores del crecimiento cuya actividad depende de la capacidad que tenga el tejido de transportarlas o de convertirlas en otro compuesto activo. Como otras fitohormonas, las giberelinas tienen un papel primordial en múltiples procesos como el desarrollo de la semilla, la elongación del tallo y el control del tiempo de floración. Estas hormonas están sujetas a una compleja regulación, ya que reprimen la expresión de múltiples genes cuyos productos están relacionados con su biosíntesis y promueven la expresión de otros genes relacionados en su inactivación (Santner *et al.*, 2009).

4.4 ETILENO

El gas etileno se sintetiza a partir de la metionina en la mayoría de los tejidos de la planta, comúnmente en respuesta a un estrés. Normalmente las mayores tasas de

producción de etileno aparecen en tejidos senescentes y en fruto durante la maduración (Figura 12). Dado que es un gas, el etileno se mueve por difusión desde los lugares de síntesis. Un precursor crucial en la biosíntesis del etilenos es el ácido 1-aminociclopropano-1-carboxílico (ACC), la adición de este propicia la síntesis del mismo y puede ser transportado produciendo efectos a distancia.

El etileno es tradicionalmente conocido por su papel en la maduración del fruto y aunque sea en pequeñas cantidades se ha relacionado también con un crecimiento anormal de las plantas. Este es producido por las propias plantas y tiene como función regular su crecimiento. Los efectos fisiológicos producidos por el etileno en cultivos son idénticos a los producidos por las auxinas, ya que se ha demostrado que un tratamiento con auxinas aumenta la biosíntesis de la enzima ACC sintasa. Por ello se cree que algunas de las respuestas de las planta a las auxinas realmente están provocadas en última estancia por el etileno producido por estas.

ETILENO	EFECTOS DEL ETILENO
$CH_2=CH_2$	• Mantenimiento del gancho apical en plántulas • Estimulación de respuestas de defensa • Crecimiento y diferenciación de raíz y parte aérea • Formación de raíces adventicias • Abscisión de flores y frutos • Inducción floral en algunas plantas • Senescencia de hojas y flores • Maduración de frutos

Figura 12. Procesos fisiológicos en los que interviene el etileno y estructura molecular.

4.5 ÁCIDO ABSCÍSICO (ABA)

El ABA es una sustancia natural presente en los tejidos vegetales que se sintetiza a partir de gliceraldehido-3-fosfato a través del isopentenyl difosfato y carotenoides (Figura 13). Los principales lugares de biosíntesis son las raíces y las hojas maduras, fundamentalmente como respuesta a un estrés hídrico. Las semillas suelen ser también ricas en ABA importado desde las hojas o sintetizado *in situ*. El ABA se exporta desde las raíces a través del xilema y desde las hojas a través del floema. Algunas evidencias apuntan a que el ABA podría circular hacia las raíces en el floema y volver hacia la parte aérea vía xilema.

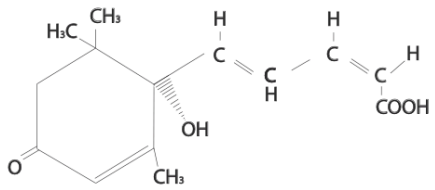
ÁCIDO ABSCÍSICO	EFECTOS DEL ÁCIDO ABSCÍSICO
	• Cierre estomático • Inhibición del crecimiento de la parte aérea • Síntesis de proteínas de reserva en semillas • Inducción y mantenimiento de la dormancia en semillas • Defensa frente ataques de insectos

Figura 13. Principales procesos fisiológicos sobre los que interviene el ácido abscísico y estructura molecular.

El ABA se ha identificado como un inhibidor del crecimiento vegetal, ya que puede controlar la dormancia en yemas y semillas. El ABA tiene otros papeles reguladores en plantas como la regulación de la clausura del estoma, control de la absorción de agua e iones por parte de las raíces y la abscisión de la hoja y la senescencia. En cultivo de tejidos algunas veces puede estimular la morfogénesis o inhibir el crecimiento del callo.

4.6 ÁCIDO JASMÓNICO

Otro grupo importante de hormonas vegetales son los jasmonatos. Están representados principalmente por el ácido jasmónico y su metil éster. El ácido jasmónico se sintetiza a partir del ácido linolénico. Este grupo de compuestos tiene un papel muy importante en las respuestas de las plantas ante condiciones de estrés. Asimismo, inhiben el crecimiento de la planta y la germinación de las semillas, promueven senescencia y maduración de frutos, y favorecen la formación de pigmentos.

El ácido jasmónico y sus metabolitos, colectivamente conocidos como jasmonatos, son moléculas de señalización endógenas muy importantes que median en respuesta tanto a estreses bióticos o abióticos como también en aspectos del crecimiento y desarrollo de la planta. El ácido jasmónico es una molécula de respuesta rápida, actuando inmediatamente después de producirse heridas mecánicas o efectuadas por herbívoros y también como respuesta a otros estreses abióticos (Santner *et al.*, 2009).

4.7 ÁCIDO SALICÍLICO

El ácido salicílico, más recientemente considerado como un regulador de la fisiología de la planta, se sintetiza a partir del aminoácido fenilalanina. Sus efectos sobre la planta son variados, destacando su papel en la defensa frente estreses bióticos. Además,

también favorece el desarrollo y longevidad de la flor, inhibe la síntesis de etileno y, en algunos casos, revierte los efectos del ABA.

4.8 BRASINOESTEROIDES

Por último, los brasinoesteroides son un grupo de alrededor de 60 compuestos esteroideos, que tienen como estructura base el brasinolido, aislado por primera vez a partir del polen de *Brassica*. Actúan sobre la división y elongación celular y la diferenciación vascular, son importantes para la fertilidad, inhiben el crecimiento y desarrollo de la raíz, y pueden promover la síntesis de etileno.

5 TRANSFORMACIÓN GENÉTICA

La introducción de genes al interior de las plantas por ingeniería genética se está convirtiendo cada vez más en una técnica aceptada en mejora genética en determinados países. Genes derivados de plantas que no guardan ninguna relación entre sí e incluso de distintos reinos (Bacteria, fungi, animales) pueden ser introducidos con estas técnicas en los programas de mejora, algo totalmente inaccesible para los mejoradores clásicos (De Block, 1993).

Para realizar experimentos de transformación genética en especies vegetales se han empleado diversas técnicas que pueden dividirse en dos grandes grupos, las técnicas directas, que inducen la permeabilidad de la membrana celular al ADN y las indirectas donde la introducción del ADN en la célula se produce mediante vectores.

5.1 TÉCNICAS DIRECTAS DE TRANSFORMACIÓN

Hay diversas técnicas directas de transformación genética de plantas. Estas se basan en producir cambios en la membrana plasmática de la célula para que el ADN pueda penetrar en la célula, ya que en condiciones normales no lo haría. Estos cambios inducidos se basan en la producción de microporos en la membrana o bien aplicando campos eléctricos (electroporación), con el uso de láser o ultrasonidos; o aumentando la permeabilidad de la membrana con polietilenglicol; o bien introduciendo de forma directa el ADN en la célula con microinyecciones o técnicas de biolística. Las técnicas directas son más eficientes para usarlas en cultivos de meristemos que las indirectas (De Block, 1993).

Ejemplos de estas técnicas son:

Electroporación: En la electroporación, se aumenta la permeabilidad de la membrana y la pared celular al ADN por pulsos eléctricos (D'Halluin *et al.*, 1992). Esto se realiza modulando la intensidad de los pulsos eléctricos aplicados.

Polietilenglicol (PEG): El polietilenglicol es un polímero capaz de aumentar la permeabilidad de la membrana. Es un método que ha demostrado ser eficaz en maíz (Omirulleh *et al.*, 1993). El problema que requiere el uso de PGE en transformación es que requiere un sistema de protoplastos, lo que reduce la frecuencia de su aplicación.

Microinyección: Se basa en la aplicación microinyecciones de ADN en el interior celular, pudiendo llegar incluso al interior del núcleo (Neuhaus y Spangenberg, 1990). Aunque a priori puede usarse para todo tipo de especies vegetales tiene la desventaja de que requiere un alto grado de tecnificación y la técnica se aplica a célula a célula, lo que implica una alta especialización y un gran consumo de recursos y tiempo.

Biolística: Dentro de las técnicas directas es la que más habitualmente se usa y la más aceptada. La biolística o técnica de bombardeo con microproyectiles con ADN fue desarrollada por Sanford, Kelin, Wolf y Allen en la Universidad de Cornell en el estado de Nueva York (EE.UU.) en 1987.

La técnica consiste en bombardear una célula o tejido con partículas de 0,4 a 2 μm de diámetro recubiertas de ADN. Estas partículas están fabricadas de un metal pesado que puede ser oro o tungsteno y se recubren de ADN con plásmidos donde se localizan los genes de interés, estos microproyectiles se aceleran en un cañón o pistola y se disparan a gran velocidad sobre el material vegetal, de forma que atraviesen la pared celular, las membranas de la célula y el núcleo. Cuando la partícula ha penetrado en el núcleo de la célula, el ADN se libera, y por recombinación se inserta en el genoma celular (Figura 14). Los ajustes con esta técnica van directamente dirigidos a una mayor penetración del ADN dentro de los tejidos, esto se consigue modulando la velocidad y el tamaño de los microproyectiles.

Éste método de transformación tiene como inconvenientes el hecho de ser muy agresivo para el material vegetal, produciendo grandes daños tisulares y celulares a la vez que los porcentajes de transformación estable son escasos, debido a la baja probabilidad existente de que el ADN se inserte en los cromosomas de las células. Para mejorar los resultados con este método se debe encontrar un equilibrio adecuado entre la eficiencia de transformación y el daño tisular que produce, ya que esto dificultaría la posterior regeneración de las células en plantas viables. Otro dato a tener en cuenta es, que con este método las integraciones múltiples del gen de interés son más frecuentes y es recomendable tener células en división activa para tener un mayor éxito con esta técnica (Ye *et al.*, 1994).

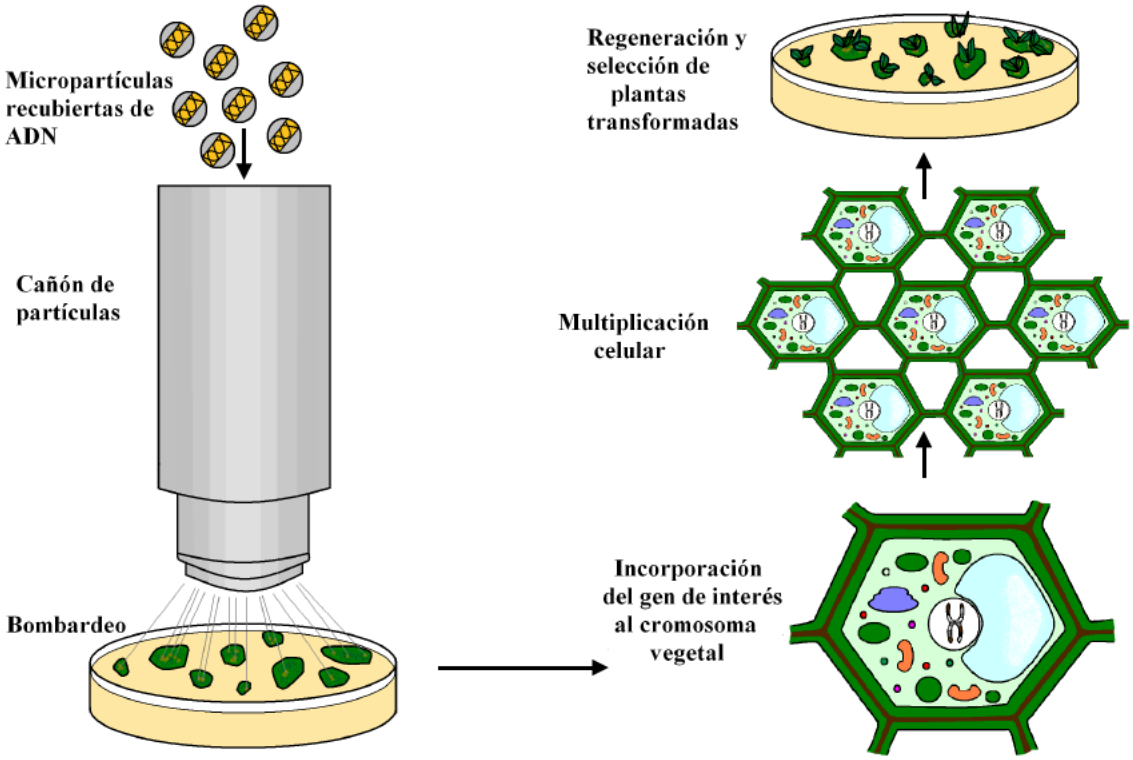


Figura 14. Técnica de biolística para la transformación de tejido vegetal.

Las ventajas que presenta esta técnica frente a otras, como las indirectas que implican el uso de bacterias como vectores, es que no hay que usar antibióticos para posteriormente eliminar la bacteria, evitando el pardeamiento y la necrosis tisular. Para la selección de células transgénicas se requiere de una cantidad mínima de agente selectivo, ya que estas células son altamente sensibles a las sustancias empleadas como agentes selectores. Otra ventaja es que con ésta

técnica se pueden usar meristemos como material de partida (McCabe y Martinelli, 1993). El objetivo es transformar células que den quimeras de las que obtener plantas transformadas en la siguiente generación. Esto reduce la etapa de cultivo *in vitro* y abre el uso de la técnica a un rango amplio de especies, importante sobre todo para aquellas que no tienen muy desarrollados protocolos *in vitro*.

En resumen, en la producción de plantas transgénicas a partir de técnicas de transformación directa el porcentaje de células que integran el gen de interés es bastante bajo. En la mayoría de los casos aparecen fragmentos de ADN insertados en lugares del genoma que deberán ser eliminados como ADN basura, ya que estos no son los más indicados para los objetivos determinados a priori.

5.2 TÉCNICAS INDIRECTAS DE TRANSFORMACIÓN

Hay determinadas técnicas en las que el ADN no se introduce directamente en la célula vegetal, sino que se hace por medio de vectores que pueden ser bacterias, virus o incluso vesículas lipídicas.

Las técnicas más representativas son:

Liposomas: Esta idea de transformación se desarrolló en los años 80 y buscaba la introducción de ADN en la célula por medio de liposomas. La técnica consiste en encapsular las vesículas con fragmentos de ADN, adherirlas a las membranas para su fusión con ellas y así transferir el ADN que las envuelve. A este fenómeno se le conoce como lipofección; el ADN penetraría en el interior celular y de ahí al núcleo. Cuando el explanto utilizado son protoplastos la lipofección se produciría a través de la fusión con la membrana o endocitosis.

La principal ventaja de esta técnica es que puede ser ampliamente utilizada en muchas especies y el equipo necesario es de bajo coste. Por contra, es una técnica muy poco utilizada debido a la alta especialización y complejidad, y su baja eficacia. De hecho se conocen muy pocos casos de transformación con esta técnica (Rao *et al.*, 2009).

Virus: Los virus fitopatógenos también han sido utilizados como vectores en experimentos de transformación genética de plantas. Son virus modificados mediante ingeniería genética de tal manera que tienen la capacidad de infectar a la planta, de autoduplicarse y de duplicar el gen o genes quiméricos de interés sin causar síntomas indeseables ni pérdidas en la producción (Agrios, 1999). Esta técnica se encuentra limitada por el tamaño del transgén y por la baja infectividad de los virus. Algunos virus utilizados han sido el virus del mosaico del tabaco (TMV) o el virus del mosaico de la coliflor (CaMV).

Agrobacterium: Aunque se han encontrado otras bacterias capaces de transformar células vegetales (*Rhizobium sp., Sinorhizobium melitoti, ;Mesorhizobium loti* (Broothaerts *et al*., 2005) *Agrobacterium* es el género para transformación más ampliamente distribuido y versátil.

El descubrimiento de *Agrobacterium* y su capacidad para introducir ADN en las células vegetales supuso una revolución en la biotecnología de plantas, ya que es uno de los métodos más eficaces de transformación genética en especies vegetales. Su éxito radica en que se trata de un organismo procariota con capacidad para introducir un fragmento de ADN en la célula vegetal y que además sus genes son reconocidos y expresados por la célula eucariota.

El género *Agrobacterium,* está formado por bacterias Gram negativas con metabolismo anaeróbico de tipo quimioheterotrofo que utilizan compuesto orgánicos como fuente de carbono y energía. Son organismos móviles con flagelación lateral cuyo hábitat natural es el suelo. Hay dos especies principales de *Agrobacterium* utilizadas para transformación, *Agrobacterium tumefaciens y Agrobacterium rhizogenes*. En la naturaleza, *Agrobacterium* infecta las partes de la planta donde se localiza una herida y hay una liberación de compuestos fenólicos al medio. *A. tumefaciens* produce una enfermedad conocida como «agallas en corona» produciendo unas estructuras similares a tumores en la base del tallo de plantas dicotiledóneas, *A. rhizogenes* provoca desarrollo de raíces.

En 1907 Smith y Townsend demostraron el origen bacteriano de la formación de las agallas y desde entonces los estudios se centraron en

determinar el mecanismo por el cual la bacteria causaba dicha enfermedad. No fue a hasta años después con el descubrimiento de las auxinas y las citoquininas cuando se descubrió que estas hormonas estaban presentes en los tumores producidos por *Agrobacterium* (Link y Eggers, 1941; Braun, 1958). Después gracias a las técnicas moleculares para el análisis de ADN se descubrió la presencia de ADN bacteriano en el interior de las células de los tumores de corona (Schilperoort *et al*., 1967). En los años 70 el plásmido presente en *Agrobacterium* fue identificado (Van Larebeke *et al*., 1974; Zaenen *et al*., 1975) y denominado «plásmido inductor de tumores» (Ti), este descubrimiento influyó en que las investigaciones se centraran en los elementos que componían dicho plásmido identificando el T-DNA (ADN transferente), un segmento específico que es transferido a la célula vegetal (Chilton *et al*., 1977; Chilton *et al*., 1978; Depicker *et al*., 1978), corroborando así la hipótesis hecha por Braun en 1958, quien aseguraba que *Agrobacterium* transfería material genético a la célula vegetal que provocaba una transformación permanente de dicha célula.

Hoy se conoce que en el plásmido Ti de *A. tumefaciens* se encuentran los genes involucrados en la aparición de los tumores, en los cambios metabólicos y también en los genes responsables de la transferencia y posterior inserción del ADN. En el interior del plásmido está el T-DNA que es la fracción de ADN que se incorpora a la célula tras la infección por la bacteria. *Agrobacterium* es capaz de modificar el metabolismo de la planta para que induzca la síntesis de unos aminoácidos conocidos como opinas. Las opinas son la fuente de nutrientes de la bacteria y son específicas para cada cepa, de forma que la planta mantiene a la bacteria cuando es infectada.

El plásmido Ti consta de diferentes regiones (Figura 15), un origen de replicación (*ORI*), una región donde se alojan los genes implicados en el catabolismo de opinas, genes que actúan en el proceso de replicación, borde derecho y el izquierdo que son esenciales para la transferencia del T-DNA, la región de virulencia (región *vir*) donde se encuentran muchos genes (genes *vir*) que son fundamentales para los procesos de síntesis del T-DNA y su transferencia a la célula vegetal, y el T-DNA. En la región T-DNA, además de los bordes izquierdo y derecho están los genes implicados en la síntesis de

opinas, el gen *ipt* para la síntesis de citoquininas y los genes *iaaM* e *iaaH* que están implicados en la síntesis del AIA. Además de los genes bacterianos incluidos en el plásmido, para que la bacteria sea capaz de infectar las células vegetales, son necesarios determinados genes cromosómicos de virulencia *chv,* implicados tanto en el movimiento quimiotáctico bacteriano como en la unión de la bacteria a receptores específicos de la célula vegetal.

En cuanto a su utilización en un proceso controlado de transformación, el uso de cepas desarmadas de *Agrobacterium* es el método más utilizado para introducir ADN en células vegetales. El proceso conlleva el desarme por deleción de las regiones que controlan la síntesis y catabolismo de opinas a la vez que las de síntesis de auxinas y citoquininas, que son las responsables de la producción del tumor. Así, se nos queda un plásmido no productor de opinas, no oncogénico y con capacidad de transformación. Ahora, por técnicas de ingeniería genética es posible introducir el gen de interés entre los bordes de la región T-DNA, de esta forma cuando ocurre la infección la célula infectada es capaz de incorporar a su ADN el gen contenido en el plásmido. El nombre que se da a una cepa normalmente hace referencia al nombre dado al plásmido Ti desarmado que porta.

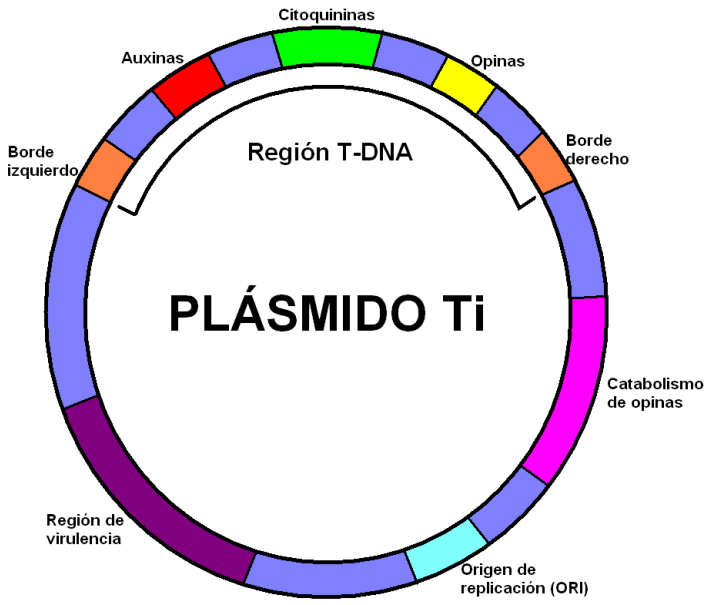


Figura 15. Estructura del plásmido Ti de *Agrobacterium.*

Hay dos tipos de vectores, aquellos que la región *vir* actúa en *cis* y los que actúan en *trans* respecto al T-DNA. Los vectores cointegrados son los que actúan en *cis*, donde un plásmido de *Escherichicia coli* se transfiere a *Agrobacterium*, el ADN foráneo se integra en los bordes del T-DNA por recombinación homóloga, dando lugar a un plásmido con una región *vir* y T-DNA con ADN foráneo. Los vectores binarios son los que actúan en *trans,* dejando por un lado está el plásmido Ti desarmado con solo la región *vir* y por otro lado un segundo plásmido con el ADN integrado entre los bordes del T-DNA. Este último mas pequeño y manipulable para introducir genes foráneos entre bordes. Estos vectores son los más utilizados debido a la mayor sencillez de su construcción.

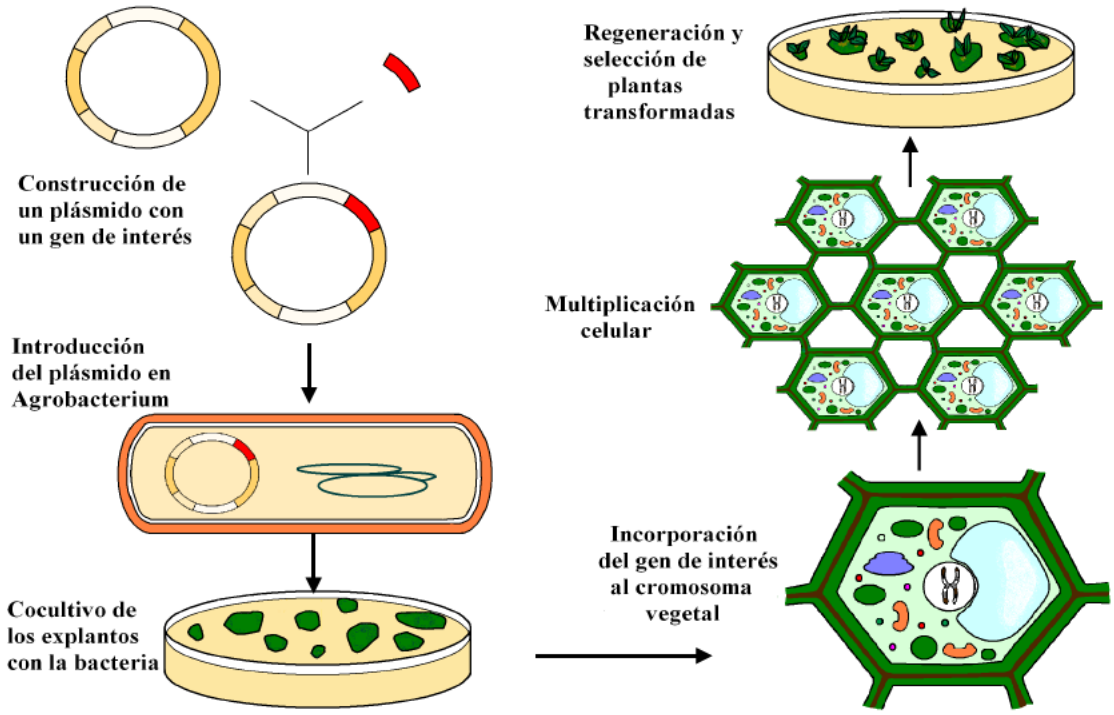


Figura 16. Técnica de transformación de tejido vegetal por bacterias del género *Agrobacterium.*

En transformación también se utiliza, aunque en menor medida, *Agrobacterium rhizogenes.* Esta bacteria contiene un plásmido denominado Ri que contiene genes implicados en el proceso de desarrollo de raíces, estos genes son conocidos como *rol* A, *rol* B y *rol* C localizados integrados en la región de T-DNA. El principal inconveniente que presenta *A. rhizogenes* con respecto a *A.tumefaciens* es que el espectro de hospedadores es más reducido.

Actualmente la metodología a seguir en *Agrobacterium* se basa en la introducción de un gen de interés en la bacteria en la región de T-DNA de su plásmido, el cocultivo del explanto y la bacteria para posteriormente eliminarla, y por último inducir la regeneración de las células transformadas (Figura 16). Un problema importante que presenta la transformación con *Agrobacterium*, es el pardeamiento y la necrosis que produce en los tejidos tras el cocultivo.

5.3 GENES MARCADORES

La eficacia de células transformadas en un evento de transformación es bastante reducida, por ello existe una serie de genes marcadores que ayudan a discriminar entre aquellas células que han incorporado el ADN foráneo y aquellas que no. Estos genes se introducen en la célula contenidos en el mismo fragmento de ADN en el que se introduce el gen de interés. Así en un cultivo es posible cuantificar y seleccionar aquellas células que se han transformado con éxito. En la actualidad hay dos tipos de marcadores, marcadores selectivos y marcadores visuales.

5.3.1 Marcadores selectivos

Se somete al explanto a un medio selectivo donde se favorece el crecimiento de aquellas células que han incorporado el marcador de selección. El agente selectivo puede ser un antibiótico o un herbicida, siendo el más comúnmente usado el gen de la neomicina fosfotransferasa (*nptII*) que ofrece resistencia mediante fosforilación a antibióticos aminoglicósidos como la kanamicina, paromomicina, neomicina o el G418. Para un mayor éxito de estos agentes selectivos es aconsejable establecer la concentración mínima para la cual estos agentes son letales para el explanto o impiden la multiplicación y regeneración del mismo, ya que las condiciones de cultivo al igual que el genotipo o el tipo de explanto pueden variar la respuesta a estos compuestos. El problema presentado muchas veces es la alta toxicidad que aportan estos agentes, ya que algunos como la kanamicina son capaces de reducir la eficiencia en el desarrollo de las mismas plantas transgénicas, otros agentes como la paromomicina presentan menor toxicidad en este sentido (Wang *et al*., 2005).

Además del gen *nptII*, se han usado, aunque en menor medida, otros como el gen que confiere resistencia a la higromicina fosfotransferasa (*htp*) y el gen de la resistencia a fosfinotricina (*bar*).

5.3.2 Marcadores visuales

Se somete al explanto a un medio al cual se le añade un sustrato donde al reaccionar con enzimas introducidas en las células transformadas producen una señal visual que las distinguen del resto. Los sustratos suelen ser cromogénicos, fluorogénicos, emisores de fotones o radiactivos.

Actualmente uno de los marcadores más usados es el GFP (Green fluorecent protein) (Shimomura, 1979), es un gen procedente de la medusa *Aequorea victoria* que codifica para una proteína que al ser excitada con luz azul emite una fluorescencia verde, con un pico de emisión a los 509 nm. Otro gen marcador ampliamente utilizado es el gen de la β-glucuronidasa (*gus*A), aislado en *E.coli*, que hidroliza β-glucurónidos. Dependiendo del sustrato donde se incuben estas células transformadas obtendremos un precipitado azul con el sustrato 5-bromo-4-cloro-3-indolil-β-D-glucurónido (X-Gluc) o un compuesto fluorescente con el 4-metil-umbeliferil-β-D-glucurónido (4-MUG).

Aunque con los marcadores genéticos se seleccionan fácilmente células y plantas transformadas, estos no suponen una prueba definitiva de que el individuo contenga el gen de interés que se pretende introducir. Por ello, para demostrar la presencia de este gen se deben realizar pruebas moleculares como una PCR (Reacción en Cadena de la Polimerasa) y especialmente la prueba de hibridación molecular Southern (Southern, 1975).

5.4 FACTORES QUE AFECTAN A LA TRANSFORMACIÓN GENÉTICA

Al igual que la regeneración, la transformación genética está afectada por numerosas variables que pueden ser tanto ambientales como genéticas.

5.4.1 El explanto

El tipo de explanto utilizado varía mucho según la especie y el protocolo. Pueden utilizarse desde tejidos inmaduros de semilla como cotiledones (Laimer da Câmara Machado *et al.*, 1992), hipocotilos (Scorza *et al.*, 1995b), hojas y callos procedentes de macizos de proliferación (Mezzetti *et al.*, 2002) entre otros. El tipo de explanto, su estado fisiológico y el genotipo tienen una gran influencia en la transformación. No todas las especies presentan la misma plasticidad para la transformación, en incluso dentro de la misma especie aparecen grandes diferencias entre líneas y genotipos. Por ello es

imprescindible ajustar las distintas variables de un protocolo de transformación al explanto y el genotipo con el que se va a trabajar.

5.4.2 La técnica

El método escogido para llevar a cabo la transformación afecta no solo al porcentaje de células transformadas sino también al mismo éxito del evento de transformación. En general la técnica más empleada entre los investigadores es el uso de *Agrobacterium tumefaciens*, aunque también en menor medida de *Agrobacterium rhizogenes* y biolística. Para cada una de estas técnicas existen un número de parámetros que pueden variar el éxito de la transformación en determinadas especies. Así por ejemplo, en el caso de la biolística variables como la distancia entre el proyectil y el tejido diana, el tamaño o la composición de los microproyectiles o la presión ejercida deben ser ajustados para establecer un protocolo. En *Agrobacterium* es importante tener en cuenta la densidad óptica de la bacteria, el tiempo de exposición del explanto a la misma, el tiempo de cocultivo, el uso y la concentración de compuestos fenólicos como la acetosiringona o el antibiótico utilizado y su concentración. La cepa bacteriana también es importante, dado que hay cepas más o menos virulentas y que presentan más o menos eficacia dependiendo de la especie vegetal con la que se esté trabajando. Así, experimentos de inoculación preliminares deberían ser realizados para comprobar la compatibilidad entre la especie hospedadora y la estirpe de *Agrobacterium* antes de llevar a cabo la transformación (Cervera *et al.*, 1998).

5.4.3 Selección de transformantes

El tipo y la concentración de agente selectivo afecta a la aparición de células y plantas transformadas. Normalmente suele utilizarse una concentración de agente que inhiba la regeneración de las células no transformadas, aportándole una ventaja a aquellas que si lo han hecho, aunque en muchos casos el agente selectivo afecta a la regeneración de las plantas transgénicas (Wang *et al.*, 2005). Para ello es necesario optimizar los protocolos y hacer una buena elección del compuesto a utilizar en un determinado material vegetal.

5.5 TRANSFORMACIÓN GENÉTICA DE MELOCOTÓN

Hasta ahora las investigaciones desarrolladas en transformación genética de melocotón no han permitido el desarrollo de un protocolo estable. Este hecho puede estar causado por la ausencia a su vez de un protocolo reproducible de regeneración somática.

Hasta la fecha, los trabajos publicados en melocotón hablan siempre de transformación desde tejidos zigóticos por la ausencia de regeneración desde material adulto. Esto conlleva el inconveniente de la modificación genética de un individuo desconocido genéticamente, debido al hecho de que una semilla es la suma de dos parentales que producen un genotipo totalmente nuevo. Aún así, podrían incorporarse genes de interés sin presencia en la naturaleza de esa especie, como resistencia a alguna enfermedad, que luego pudieran emplear en un programa de mejora como nueva fuente de germoplasma.

Algunos autores han publicado revisiones de ensayos de transformación en *Prunus* incluyendo al melocotón (Scorza y Hammerschlag, 1992; Scorza *et al.*, 1995a; Rugini y Gutierrez-Pesce, 1999; Srinivansan y Scorza, 1999; Srinivansan *et al.*, 2004), y en la mayoría de los artículos publicados el método utilizado para la transferencia de genes en células de melocotón ha sido *A. tumefaciens*, el gen marcador utilizado el *nptII,* y en algunos casos el gen *gus* como marcador visual aunque también *GFP*. Aunque la transformación en melocotón siempre se ha visto limitada por la ausencia de un protocolo de regeneración, la técnica por sí misma tiene sus propias dificultades en esta especie. Así, a pesar de que comúnmente en la naturaleza *A. tumefaciens* produce agallas en el melocotonero (Scorza y Sherman, 1996), la eficiencia de transformación en células de melocotón es relativamente baja. Hasta la fecha, solo hay dos publicaciones que indiquen el desarrollo de plantas de melocotón transgénicas (Smigocki y Hammerschlag, 1991; Pérez-Clemente *et al.*, 2004). Smigocki y Hammerschlag en 1991, fueron los primeros autores en obtener plantas de melocotón transgénicas de la variedad «Redhaven» expresando el gen *ipt* de *A. tumefaciens*. El explanto de partida fue callo embriogénico de embriones inmaduros y la cepa utilizada de *A. tumefaciens* es un mutante denominado «shooty mutant» tms328::Tn5 que contiene un plásmido con gen funcional de citoquininas y otro mutado de producción de auxinas. El uso de esta cepa mutante productora de citoquininas podría ser la responsable del éxito de la regeneración de brotes ya que los niveles de citoquininas de los brotes transgénicos eran 50 veces mayores que los de los brotes control, además las plantas transgénicas mostraron alteraciones en sus patrones de crecimiento comparadas cono los controles no transformados. Esto incluía la formación de árboles enanos, un aumento en la producción de ramas y un retraso en la caída de la hoja (Hammerschlag *et al.*, 1997; Hammerschlag y Smigocki, 1998). Estas características son propias de altas concentraciones de citoquininas y aparentemente estaban debidas a la expresión del gen *ipt* de *A. tumefaciens* en las líneas

transgénicas de melocotón. Pérez-Clemente *et al.* en 2004 también obtuvieron plantas transgénicas procedentes de tejido de semilla transformado con la cepa de *A. tumefaciens* C58, obteniendo porcentajes de regeneración que rozaban el 50% en secciones de embriones.

Scorza *et al.* (1990) transformaron segmentos de hoja de melocotón y callos embriogénicos que habían sido mantenido durante mucho tiempo con la cepa de *A. tumefaciens* A281 con el plásmido pGA472 que contenía el gen de selección *nptII*. Las tasas de transformación en los callos fue del 5 % en contraste con el 64 % que presentaron las secciones de hoja. Por contra, ninguno de los dos explantos fue capaz de regenerar por lo que no se produjeron brotes ni plantas transgénicos.

Por otro lado, también se han realizado experimentos en melocotón con técnicas de biolística en callos embriogénicos de melocotón (Ye *et al.*, 1994), que derivaban del cultivo de embriones inmaduros. Para tener un material en multiplicación activa antes del bombardeo hicieron frecuentes subcultivos en un periodo de entre 3-14 días. Partiendo de 114 líneas de callos 65 fueron transformadas y de estas 7 presentaron claras evidencias de haber sido transformadas con técnicas de PCR y análisis histológicos de GUS, pero en este ensayo no hubo regeneración por lo cual tampoco plantas transformadas.

El melocotón no es el único *Prunus* con dificultad en términos de transformación y regeneración ya que solo hay unos pocos autores que han publicado éxito en la transformación de otras especies del género *Prunus* (Tabla 3).

Tabla 3. Resumen de los trabajos de transformación publicados en distintas especies del género *Prunus*

Especie	Explanto	Método	Cepa	Genes	Referencia
Prunus armeniaca	cotiledones	*A.tumefaciens*	LBA4404	nptII, gus	Laimer da Camara Machado *et al*, 1992
	Brotes	*A.rhizogenes*	A4, C7	ADN-T (ipt), ppvCp	Escalettes *et al*, 1994
	Hojas	*A.tumefaciens*	C58GI, C58C	nptII, gus, ppvCp, hyg	
	Hojas	*A.tumefaciens*	EHA105	nptII, gfp, uidA	Petri *et al*, 2008
Prunus avium	Brotes	*A.tumefaciens*	C58	nptII, gus	Brasileiro *et al*, 1991; Gutierrez-Pesce *et al*, 1998
	Brotes	*A.rhizogenes*	NCPPB 1855	ADN-T (ipt)	
Prunus avium × *Prunus cerasus*	Hojas	*A.tumefaciens*	EHA105	nptII, gus	Dolgov y Firsov, 1999
Prunus avium × *Prunus pseudocerasus*	Brotes	*A.rhizogenes*	NCPPB 1855	ADN-T (ipt)	Gutierrez-Pesce *et al*, 1998
Prunus davykensis	Brotes	*A.rhizogenes*		ADN-T (ipt), bar	Druart *et al*, 1998
Prunus domestica	Hipocotilos	*A.tumefaciens*	EHA101	nptII, gus	Mante *et al*, 1991
	Hipocotilos	*A.tumefaciens*	EHA101	nptII, gus, ppvCp	Scorza *et al*, 1994
	Hipocotilos	*A.tumefaciens*	C58	nptII, gus, ppvCp	Scorza *et al* 1995a, 1995b
	Hipocotilos	*A.tumefaciens*	LBA4404, EHA105	nptII, gus	Padilla *et al*, 2006
	Brotes	*A.rhizogenes*	C7	ADN-T (ipt), ppvCp	Escalettes *et al*, 1994
	Hojas	*A.rhizogenes*	C58GI	nptII, gus	
		A.rhizogenes	C58C	nptII, gus, ppvCp, hyg	
	Hojas	*A.tumefaciens*	LBA4404	nptII, gfp	Yancheva *et al*, 2002
	Ejes embrionarios	*A.tumefaciens*	LBA4404, EHA105	nptII, hpt, gus	Tian *et al*, 2009
Prunus dulcis (Prunus amygdalus)	Hojas	*A.tumefaciens*	LBA4404	nptII, gus	Archilletti *et al*, 1995
	Hojas	*A.tumefaciens*	EHA105	nptII, gus	Miguel y Oliveira, 1999
	Hojas	*A.tumefaciens*	LBA4404, EHA105	nptII, gus	Ansley *et al*, 2002
Prunus incisa × *serrula*	Callos embriogénicos	*A.tumefaciens*	LBA4404	nptII, gus	Druart *et al*, 1998
	Brotes	*A.rhizogenes*	ATCC	ADN-T (ipt), bar	
Prunus mume	Cotiledones	*A.tumefaciens*	EHA101	nptII, gus	Gao *et al*, 2010
Prunus salicina	Hipocotilos	*A.tumefaciens*	EHA105, GV3101, LBA4404	nptII, gfp	Urtubia *et al*, 2008
Prunus serotina	Hojas	*A.tumefaciens*	EHA105	nptII, AG	Liu y Pijut, 2010
Prunus subhirtella	Callos de peciolos de hojas	*A.tumefaciens*	LBA4404	nptII, gus	da Camara Machado *et al*, 1995

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CHAPTER I: EMBRYO CULTURE

1 INTRODUCTION

Peach breeding programs seek to solve different problems, namely diseases, production or postharvest problems. But breeders have also varied objectives such as adaptation of new cultivars to growing areas, disease resistance, ripening fruit, high production and organoleptic quality. Spain has a wide commercial calendar, from April to November; hence, many varieties, with its different harvest period and requirements, are needed.

Commercially, the sooner a peach is produced the higher its economic value is. Thus, the most attractive feature in a new cultivar is an early ripening fruit (Anderson and Byrne, 2001). Nevertheless, a short period between blooming and ripening leads to one problem: a low percentage of germination. This is due to the fact that an early ripening produces small immature seeds and embryo abortion when the fruit is ripe (Tukey, 1933) or during development in certain interspecific hybridizations. This problem may be caused by abortion and/or endosperm degeneration due to inherent nutritional deficiency or if they are the result of distant hybridization (Sharma *et al*., 1996). The percentage of survival is directly related to the embryo size. Attempts to solve this issue by embryo rescue have been made since 1904 (Hannig, 1904).

Tissue culture techniques have been used in the last decades in order to rescue certain genotypes which in natural conditions would not germinate. The experiments conducted by Tukey in 1933 were pioneer in developing embryo rescue techniques in stone fruit, and Blake, in 1939, applied these methods to peach breeding. In the case of stone fruit there is one physical factor, which is essential in the success of embryo culture: the chilling treatment. Thus, if embryos do not receive an accurate stratification, several problems arise such as decrease of vitality, lower germination rates, rosette-type growth and a quick pass into a dormancy stage. In order to avoid these effects, it is essential to set a temperature of around 4 ºC for, at least, 30 days after the transfer of the embryos into a nutrient medium. Hence, they form normal growing seedlings.

The media most used are Murashige and Skoog (Murashige and Skoog, 1962) and woody plant medium (Lloyd and McCown, 1980) deprived of plant growth regulators. However, there are other factors which also have an influence in embryo culture. Plant

growth regulators, carbon or nitrogen sources are involved in the development and growth of the embryos during the fruit evolution (Bassi and Ryugo, 1990). Accordingly with previous research, sucrose, fructose, glucose and sorbitol are the most commonly used sugars to serve as a carbon source and osmotic stabilizer. Although, the concentration of them is more important for the osmolarity of the medium than of providing nutrition (Raghavan and Torrey, 1964).

Nevertheless, successful embryo rescue not only depends on the culture media but also on the developmental stage of embryo and culture conditions (Chaparro and Sherman, 1994) such as photoperiod or temperature. Commonly, conditions of 24 hours in darkness during stratification and 16 hours in light at 25 ºC in climatic chamber after chilling treatment, are used. Gibberellic acid could be applied to reduce the time of germination in stone fruit seeds.

The objectives in the current study are to determine which carbon source and at which concentration is more suitable for hybrid embryos coming from a peach breeding program and whether the presence or absence of gibberellic acid during different periods of stratification improve the germination process.

2 MATERIAL AND METHODS

2.1 PLANT MATERIAL

Twelve different assisted crosses were performed in the field using early ripening peach trees as females. During April and May 2010 a total of 2,660 well developed fruits were collected and stored at 0 ºC ± 1 ºC for a period of 18 hours before its use. The stage of development varied from 50 to 70 % fill of the seed coat at time of harvesting. Seeds were first removed from the endocarp and surface sterilized in a solution of 2 % (*v*/*v*) sodium hypochlorite and 0.1 % (*v*/*v*) Tween 20 for 1.5 hours in a laminar flow hood. After disinfection, seeds were transferred to a laminar flow cabinet to remove the coats. In order to minimize the genotype effect, the seeds were distributed randomly in the three assays.

2.2 CARBON SOURCE TREATMENTS

2.2.1 Treatments

From the total of collected fruits, 1,130 seeds were randomly cultivated in 9 different media composed of woody plant medium (WPM), 0.7 % (*w/v*) of plant propagation agar (Pronadisa®) and 15, 30 or 45 g $l^{-1}$of either sucrose, sorbitol or glucose. The culture medium was poured in sterilized tubes of borosilicate glass (10 ml/tube). The pH was adjusted at 5.7 with KOH (0.1 N) prior to autoclaving for 16 minutes at 1.1 kg cm^{-2} (122 ºC). The tubes were kept in climatic chamber under 4 ± 1 ºC and darkness for 90 days.

2.2.2 Culture and data collection

When stratification was completed, lengths of roots and stems were measured. The seeds were moved to climatic chamber under 25 ± 1 ºC and 16h light (45 μmol m^{-2} s^{-1}) GRO-LUX, provided by Sylvania. 10 days after moving seeds into climatic chamber data from roots and stems were recorded and plants were transferred to pot. Both the number of germinated seeds and the percentage of germination were also recorded.

2.3 GIBBERELLIC DIP AND STRATIFICATION TREATMENTS

2.3.1 Treatments

As in the first assay 1,130 seeds were used. The seeds were sunk in a solution of gibberellic acid (GA_3) at concentrations of 0, 10 or 50 g l^{-1} for 5 minutes. The GA_3 solution was prepared in distillate water which was sterilized by filters throughout 0.22 μm nitrocellulose filter (Millipore®). Thereafter, the seeds were transferred to WPM 0.7 % (*w/v*) of plant propagation agar (Pronadisa®) and 30 g l^{-1} of sucrose. The tubes were kept in climatic chamber under 4 ± 1 ºC and darkness for 0, 15, 30 or 45 days.

2.3.2 Culture and data collection

After chilling treatment seeds were moved to climatic chamber at 25 ± 1 ºC and 16 h light (45 μmol m^{-2} s^{-1}) GRO-LUX, provided by Sylvania. Data were collected 37 days after transferring the seeds to climatic chamber. The percentage of germination was taken into account, as well as the roots and stems length.

2.4 GIBBERELLIC TREATMENTS

2.4.1 Treatments

Four hundred seeds were used for testing the effects of the inclusion of GA_3 in the culture media. Seeds were cultured randomly in WPM, 0.7 % (*w/v*) of plant propagation agar (Pronadisa®) and 30 g l^{-1} of sucrose with 0, 0.5, 1, and 2 g l^{-1} of GA_3. The tubes were kept in climatic chamber under 4 ± 1 ºC and darkness for 90 days and the roots and stems length was measured.

2.4.2 Culture and data collection

After stratification, seeds were moved to climatic chamber under 25 ± 1 ºC and 16h light (45 μmol m^{-2} s^{-1}) GRO-LUX, provided by Sylvania. Data were recorded after 10 days, including percentages of germination as well as roots and stems length.

2.5 DATA ANALYSIS

All data were subjected to ANOVA and the significance ($P \leq 0.05$) of differences between mean values was tested using Duncan's new multiple range test. Data were analysed using different applications of the SPSS software (SPSS 15.0®, Chicago, IL).

3 RESULTS AND DISCUSSION

3.1 CARBON SOURCE TREATMENTS

High percentages of germination were accomplished for the main part of the treatments, with most of them exceeding 80 % of germinated seeds (Table 1). Only the media with glucose at a concentration of 30 g l^{-1} and 45 g l^{-1} obtained a medium-low percentage of germination, with 61.25 % and 53.26 % each. Sorbitol and sucrose at a concentration of 15 g l^{-1} reached the highest rates with more than 90 % of germinated seeds.

The length of stems after stratification was higher in the seeds grown in the media with 15 g l^{-1} of glucose or 15 g l^{-1} of sucrose. The same results were obtained in the length of roots, where also the media with 15 g l^{-1} of sorbitol induced a good development. Hence, the best results in every carbon source, regarding to germination and length of stems and roots, are always achieved in media with 15 g l^{-1}. During the first stage, embryos

are using the nucellus and endosperm as well as the nutrient medium for growth (Ramming, 1990). Thus, embryos do not have a high dependency of an external source of carbon. The germination rate and the development of the organs decreased in general with higher levels of the carbon source in this experiment. Cells recognize sugars as chemical signals and a high concentration may act *in vitro* as a stressing agent (Da Silva, 2004).

In contrast, after 10 days under 25 ± 1 °C and 16h light, the roots and stems elongation were higher in plants placed in the medium with 30 g l^{-1} of sucrose. There were not significant differences between concentrations in glucose. Also, the lengths of the organs in the seeds germinated in sorbitol were lower than that of those germinated in the other two carbohydrates.

Table 1. Effect of different concentrations and carbon sources in seed germination and plant development after the stratification process and after 10 days in climatic chamber.

Carbon source	**g l^{-1}**	**%germination**	**Length (cm)**					
			Stem 1	Root 1	Stem 2	Root 2	Δ Stem	Δ Root
Glucose	15	86.15a	0.52a	1.91a	1.88	6.81	1.36ab	5.00abc
	30	61.25b	0.42ab	1.41a	1.77	7.69	1.35ab	6.29abc
	45	53.26b	0.19b	0.75b	1.54	4.96	1.35ab	4.21abc
Sucrose	15	92.75a	0.60a	1.60a	1.75	8.33	1.15ab	6.73ab
	30	82.49a	0.44ab	1.76ab	1.86	9.64	1.42ab	7.88ab
	45	89.95a	0.41ab	1.50ab	1.64	6.08	1.23ab	4.58abc
Sorbitol	15	95.10a	0.35ab	1.88a	1.42	5.85	1.07abc	3.98bc
	30	87.22a	0.22b	1.48ab	1.30	5.41	1.08bc	3.92bc
	45	83.59a	0.19b	1.25ab	0.95	3.84	0.76c	2.59c

Values in each column followed by the same letter are not significantly different ($P \leq 0.05$). Root1 and Stem1 indicate the collected data after stratification; Root2 and Stem2 refer to the collected data after 10 days in climatic chamber; ΔStem and Δroot represent the elongation of stems and roots in climatic chamber.

During the plant elongation, plants *in vitro* require an external source of carbohydrates, a factor that may compensate the lower availability of water for the plant. During sterilization sucrose is hydrolysed into fructose and glucose (Yoshida *et al.*, 1973)

and the combination of these two carbohydrates may be producing a better development of the *in vitro* plants.

Sorbitol produced a high percentage of germination but its effects in the development and elongation of roots and stems was low after stratification and before acclimatization. According to previous reports (Scozzoli and Pasini, 1991; Sinclair and Byrne, 2003) sorbitol is not suitable to induce embryo enlargement and good percentages of growing seedlings. This fact could be associated to a poor utilization of sorbitol by peach seeds and seedlings due to a low presence of enzymes involved in the hydrolysis of sorbitol into fructose and glucose. Most of the enzyme sorbitol oxidase, which converts sorbitol to glucose, is produced in the seed coat (Yamakazi and Ryugo, 1986), so the availability of this enzyme is reduced in the present experiment.

3.2 GIBBERELLIC DIP AND STRATIFICATION TREATMENTS

After 37 days, high germination rates were obtained in all the treatments, even in the control, in which no GA_3 dip was applied (Figure 1). Notwithstanding the physiological state of the plants showed large differences (Table 2) as regards to roots and stems lengths. The longer the stratification period was, the higher development in plants was obtained, before reaching a limit in which no improvements were made. In terms of stems length there were almost no differences between 30 and 45 days of stratification treatments, meanwhile roots were longer after 45 days of stratification than after 30 days. The positive effects of stratification had been previously reported by Taiz and Zaiger (2002), who affirmed that cotyledons have the ability to inhibit growth in peach but this can be overcome by cotyledon removal or chilling treatments.

In contrast, dips in GA_3 produced a negative effect on germination. High concentration of GA_3 may have produced a phytotoxic effect in seeds. Since GA_3 concentrations used in dips were high (10-50 mg l^{-1}) and each tube contained only 10 ml of medium, the final amount of GA_3 produced an inhibitory effect in seeds germination. In this case, dipping seeds in a highly concentrated GA_3 solution (50 mg l^{-1}) produced an undesirable effect. Not only low rates of germination were obtained but also shorter roots and stems in the majority of the germinated seeds. This toxicity could be overcome by stratification. Thus, the toxicity is counteracted as the days of stratification are increased.

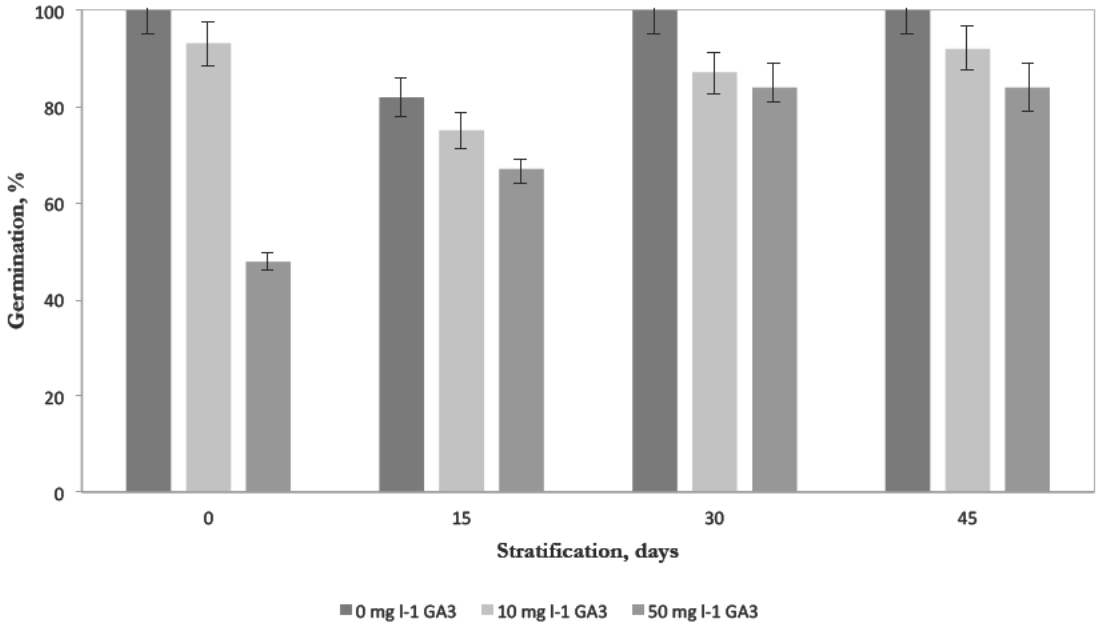

Figure 1. Effects of gibberellic acid pulses and different periods of stratification in seed germination.

Table 2. Effect of different periods of stratification and gibberellic acid pulses in roots and stem development after seed germination.

Days at 4ºC	Pulse of GA_3 mg l^{-1}	Length (cm)	
		Stem1	Root1
0	0	1.71b	1.83c
	10	1.12c	0.8d
	50	0.71d	0.93d
15	0	1.58bc	1.78c
	10	1.87b	1.56c
	50	1.34c	1.84b
30	0	2.01ab	3.08a
	10	1.82b	2.63b
	50	1.67b	2.72b
45	0	2.69a	3.57a
	10	2.83a	2.89a
	50	1.85b	2.73a

Values in each column followed by the same letter are not significantly different ($P \leq 0.05$). Root1 and Stem1 indicate the collected data after stratification

3.3 GIBBERELLIC TREATMENTS

GA_3 added to media slightly increased germination rate (Figure 2) and produced more developed organs in peach seedlings, in conditions of stratification and under photoperiod (Table 3). After stratification, the measures of roots and stem were longer in the media with 2 mg l^{-1} of GA_3. As it has been reported by Jeengool and Boonprakob (2004) seedling length increases to the amount of GA_3, known to control and enhance cell elongation and hence made the distinctive difference in length of those treated with GA_3. Gibberellins have been reported to overcome dormancy (Bridgen, 1994) favouring an earlier germination. After dormancy, sensitivity to gibberellins is increased in seeds, and they induce hydrolysis of the substances present in the endosperm. According to Sharma *et al.* (1996) one of the causes of embryo abortion is endosperm degeneration. GA_3 may be acting preserving and mobilizing its reserves putting them available for the plant. Results obtained in climatic chamber were also better in the medium with higher GA_3 content reaching measures between 5 and 6 cm long. Length in roots and stem are increasing according with GA_3 as well, avoiding undesirable phenotypes as rosette-type growth (Jeengool and Boonprakob, 2004).

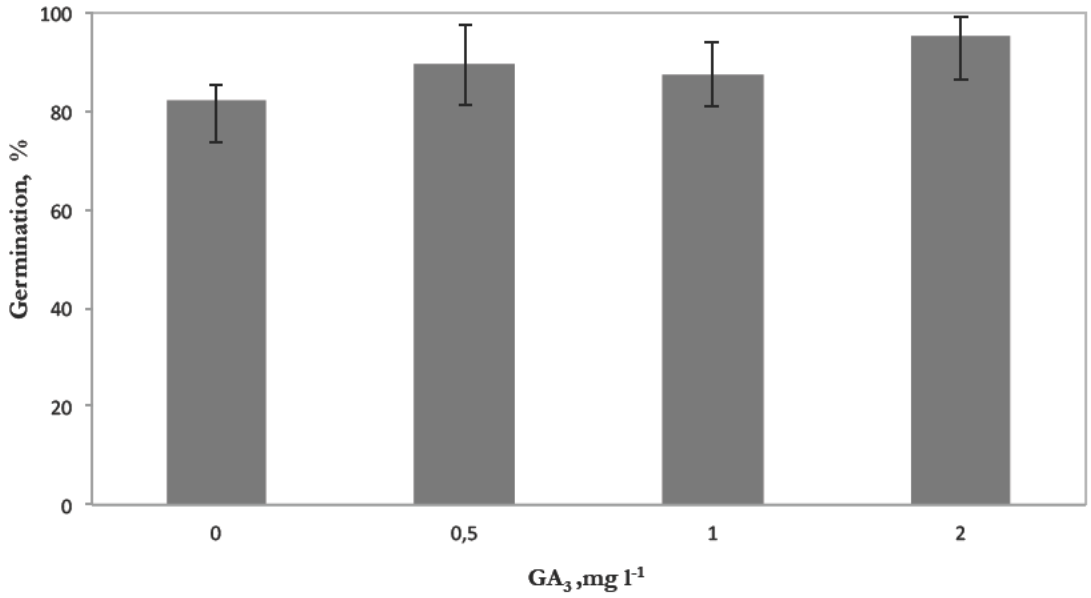

Figure 2. Seed germination in different concentrations of gibberellic acid.

The results presented here indicated that stratification is a prerequisite in the *in vitro* culture of ovules in peach. Low temperatures have a beneficial effect in germination and

also in the process of plant development, inducing an adequate roots and stems growth. Referring to carbohydrates, low concentrations of sugars benefit root and stem development during stratification. In contrast, medium concentrations of sugars induce a better elongation in roots and stems during plant growth. Glucose obtained better results in stratification and glucose during growth stage. Gibberellic acid added to the media enhanced organs development and elongation significantly, but not when gibberellic acid was provided by soaking the seed before culture. These findings suggest that, in peach, a protocol of *in vitro* embryo rescue based in two stages (stratification and culture) could enhance the quality and the amount of seedlings obtained, if each stage is provided with the adequate factors for a proper development, presented in this study.

Table 3. Effect of different concentrations of gibberellic acid (GA_3) in roots and stem development after seed germination.

GA_3 mg l^{-1}	Length (cm)					
	Stem 1	Root 1	Stem 2	Root 2	Δ Stem	Δ Root
0	1.88c	1.89b	6.15	5.26	4.27b	3.37c
0.5	2.50b	1.97b	7.51	5.98	5.01a	4.01ab
1	3.07a	2.29a	8.63	6.54	5.56a	4.28ab
2	3.39a	2.81a	9.35	8.05	5.96a	5.24a

Values in each column followed by the same letter are not significantly different ($P \leq 0.05$). Root1 and Stem1 indicate the collected data after stratification; Root2 and Stem2 refer to the collected data after 10 days in climatic chamber; ΔStem and Δroot represent the elongation of stems and roots in climatic chamber.

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CHAPTER II: CALLUS INDUCTION

Results concerning this current chapter have been accepted for publication as ***In vitro* callus induction from adult tissues of peach (*Prunus persica* L. Batsch)** in ***In Vitro Cellular and Developmental Biology Plant*** by September 5, of 2012**.** DOI: 10.1007/s11627-012-9466-8 (see Annex II).

1 INTRODUCTION

Callus is an amorphous and dedifferentiated tissue composed of disorganized cells. It may be produced naturally in response to insect or microorganism attack or stress (George 1993). Several *in vitro* biotechnological techniques have been developed, all of which require a reliable protocol to produce a responsive cell mass. Unorganized cells of *Prunus* spp. have traditionally been cultured for protoplast fusion of different individuals, somatic hybridization (Hidano and Niizeki, 1988), to obtain haploids (Peixe *et al.*, 2004), or for induction of tolerance to low temperatures (Arora and Wisniewski 1995). Callus induction has also been used for genetic transformation (Scorza *et al.*, 1994) and adventitious regeneration (Gentile *et al.*, 2002), which is the initial phase in a transformation protocol.

Peach is one of the most widely consumed fruits in the world, but its recalcitrance in many biotechnological processes has hindered the advance of *in vitro* techniques. For most purposes, *in vitro* callus establishment is important as an intermediate step in peach biotechnology. Most of the advances made in peach have used embryo-derived explants. The main disadvantage of developing a protocol from seed-derived material is that each genotype is unique and not a clone of the parent (Abbott *et al.*, 2008). Development of these biotechnological tools from mature tissues is important for the improvement of desirable commercial cultivars that have been selected for beneficial features, which may not be present in naturally produced seeds. Only a few authors have developed somatic regeneration protocols using adult peach material (Gentile *et al.*, 2002; Pérez-Jiménez *et al.*, 2012).

Many factors affect callus induction as well as its growth and development *in vitro*, namely, the type of explant tissue, quality and type of light and photoperiod conditions, plant growth regulators, culture media, gelling agent, pH, temperature, and many others. Plant growth regulators particularly influence callus induction; a phase in which auxins play a major role by inducing callus proliferation and development (Paris *et al.*, 2004). Different types of callus are frequently developed for different purposes. A white and globular callus

has been used for embryogenesis (López-Pérez *et al.*, 2005; Hammerschlag *et al.* 1985) while green and nodular calli are used for organogenesis (Gentile *et al.*, 2002; Pérez-Jiménez *et al.*, 2011). Thus, a thorough study of all the factors involved is important for determining the choice of protocol for the type of callus required.

This work aimed to develop an efficient and reproducible protocol for obtaining callus from adult material in peach, by comparing different types of explant, culture media, growth regulators, and culture conditions. The results will be useful to develop further studies, to regenerate plants, protoplast cultures, and to obtain metabolites from *in vitro* cultures.

2 Material and Methods

2.1 PLANT MATERIAL

Plant tissues were collected from 4-year-old peach (*Prunus persica* L. Batsch) trees grown at the Torreblanca experimental field station of the Instituto Murciano de Investigación y Desarrollo Agrario y Alimentario (IMIDA), Murcia, Spain. Three commercial varieties EarlyMay® (nectarine), UFO-3® (flat peach), and ZiseMay® (peach) and another three preselected varieties from a peach breeding program using hybrid crosses PS108 (EarlyMay® x N-292), PS208 (EarlyMay® x UFO-3®), and PS708 (ZiseMay® x EarlyMay®) were used as explant sources. The plants were watered daily using a drip irrigation system with 700 m^3 ha^{-1} per month at the time of sampling. The samples for callus induction experiments were taken from young branches producing their first shoots after flowering in April 2010.

2.2 *IN VITRO* ESTABLISHMENT

Branches and flowers in the balloon stage from the selected genotypes were disinfected in a solution of 2 % (*v*/*v*) sodium hypochlorite and 0.1 % (*v*/*v*) Tween 20 for 2 h. After disinfection, the plant material was transferred to a laminar flow cabinet to separate the plant organs. Six sections of vegetative organs (fully expanded leaf blade, petiole, juvenile leaf, stem, terminal buds, and axillary buds) and three reproductive organs (calyx, petals, and stamens) were selected and cultured as follows: leaf explant with the abaxial leaf surface facing the medium, with and without midvein; leaf explant with the

adaxial leaf surface facing the medium, with and without midvein; approximately 1 cm long stem explants plated in an upright or inverted position; juvenile leaf; terminal bud; axillary bud; petiole; calyx; petals; and stamens (Figure 1).

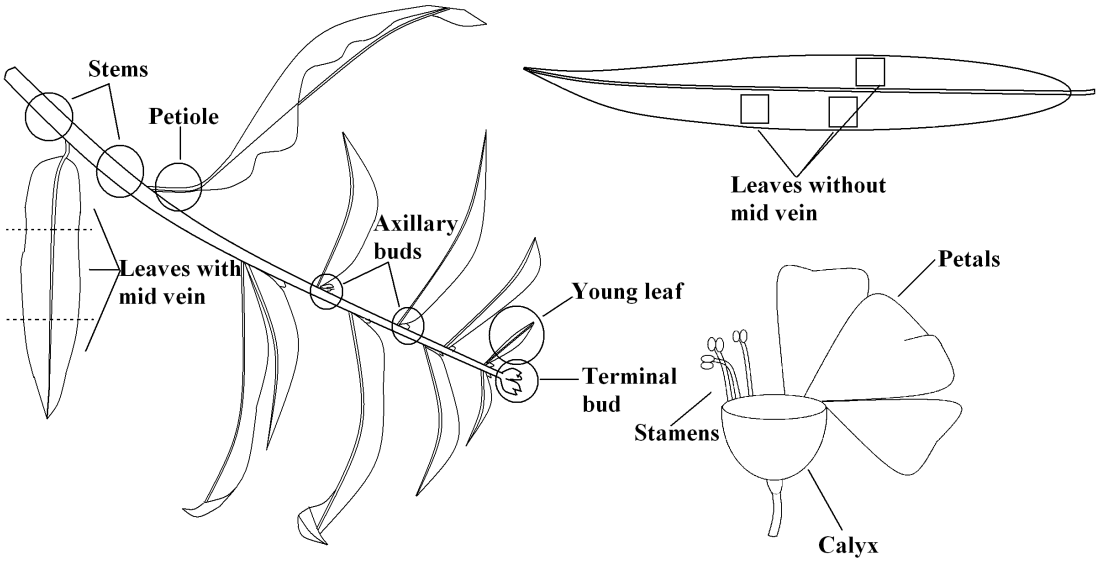

Figure 1. Source of explants used for *in vitro* callus induction of peach.

2.3 CULTURE MEDIA

The explants were cultivated on 4 different media: Murashige and Skoog medium (MS; Murashige and Skoog 1962) supplemented with 1.2 mg l^{-1} of 2,4-dichlorophenoxyacetic acid (2,4-D), and 1 mg l^{-1} of kinetin (KN, MS-DK); MS supplemented with 1.2 mg l^{-1} of 2,4-D and 1 mg l^{-1} thidiazuron (TDZ; MS-DT); Woody plant medium (WPM; Lloyd and McCown 1980) with 1.2 mg l^{-1} of 2,4-D and 1 mg l^{-1} of KN (WPM-DK); and WPM supplemented with 1.2 mg l^{-1} of 2,4-D and 1 mg l^{-1} TDZ (WPM-DT). All media contained 3 % (*w*/*v*) sucrose and 0.7 % (*w*/*v*) of plant propagation agar (Pronadisa®), in Petri dishes (12 mm ø). The pH was adjusted to 5.7 with KOH (0.1 N) prior to autoclaving for 16 min at 1.1 kg cm^{-2} (122 °C).

2.4 CULTURE CONDITIONS

The cultures were incubated in a climatic chamber at 25 ± 1 °C with two different light regimes. Half of the plates (selected randomly) with the same type and number of explants were kept under 16 h light (45 μmol m^{-2} s^{-1}, GRO-LUX, Sylvania) photoperiod, and the other half in constant (24 h) darkness.

2.5 EXPERIMENT DESIGN AND DATA COLLECTION

Data were collected from 28,080 explants. Four media were tested with 15 explants for each of the 13 types, 6 genotypes, and 2 different light conditions with three replications. During the study the following parameters were measured: percentage of explants in which callus was induced, days to the appearance of callus, color and type of callus produced, and callus relative growth (CRG) – which refers to the percentage of explant covered by callus. Data were recorded each day to know exactly when callus started to appear, and at 30 d, CRG was recorded, as well as the number of explants and which part of the explant that the callus growth originated. No sub-cultures were performed during the experiment.

Significance was determined by analysis of variance (ANOVA) and the least significance ($P \leq 0.05$) differences among mean values were estimated using Duncan's new multiple range test.

3 RESULTS AND DISCUSSION

Callus was induced in all six genotypes of *P. persica* cultivated on four different media supplemented with 2,4-D, KN, or TDZ and 2 light regimes: 16 h light and constant darkness. Disinfection resulted in effective prevention of contamination and only 1.4 % of explants died from the process. No significant genotypic differences were observed for any of the three parameters assessed for the eight different treatments (Table 1). A high percentage of callus formation was demonstrated for all the six genotypes, where the percentage of explants developing callus ranged from almost 80 % in PS208 to a maximum of 91 % in PS708. Generally, explants started to develop callus from 14 to 16 days after the experiment commenced (Table 1). The amount of callus induced was statistically the same

in all the studied genotypes therefore further studies in callus induction did not discriminate between peach genotypes.

Table 1. Percentage of explants developing callus, beginning of callus induction (Days) and the callus relative growth (CRG) on callus induction of six peach genotypes.

Cultivar	Explants developing callus (%)	Days	CRG (%)
PS708	91.02a	15.3a	38.09a
PS108	84.16a	15.4a	38.40a
PS208	80.96a	15.8a	40.28a
UFO-3®	83.86a	15.7a	38.95a
Early May®	87.12a	16.4a	42.36a
Zise May®	90.04a	16.1a	41.29a

Values in each column followed by the same letter are not significantly different ($P \leq 0.05$)

Thirteen treatments with a variety of explant types were tested for callus induction (Table 2). Statistical differences were observed between groups; however, all the genotypes provided a high level of callus induction for the different explants (above 80 %), and all responded in a similar timeframe. The only exception was the stamen explants which were completely unresponsive to all media and conditions tested. Due to the small standard deviation, there were three statistical groups: the stamens and the other two in which the differences between both had a small statistical significance. The lack of response from anthers was unexpected since callus induction from anthers has been reported previously in *Prunus* species. Long *et al.* (1994) and Peixe *et al.* (2004) obtained callus from anthers in *Prunus avium* and *Prunus armeniaca*, respectively. Both authors used culture media different to the ones used in these experiments, with the exception of Peixe *et al.* (2004), who also used MS salts, although their best result was obtained with Nitsch and Nitsch medium. As regards the percentage of explants that produced callus, the rates ranged from 82.37 % to 98.96 %, and the average time when the callus started to develop ranged from 14.4 to 17.3 d, similar to the results obtained in almond by Işikalan *et al.* (2010).

Table 2. Influence of thirteen different explant sources on callus induction of six genotypes of peach.

Explant	Explants developing callus (%)	Days
Ab-Leaf WV	85.65b	17.2b
Ab-Leaf WoV	83.37b	17.3b
Ad-Leaf WV	85.52b	16.3b
Ad-Leaf WoV	96.44c	16.6b
Axillary bud	96.50c	14.8a
Calyx	98.96c	14.5a
Terminal bud	97.19c	16.3b
Juvenile leaf	82.37b	16.2b
Petals	97.48c	16.2b
Petiole	97.69c	16.3b
Stamens	0.00a	-
Stem-up	98.50c	14.7a
Stem-in	97.04c	14.4a

Values in each column followed by the same letter are not significantly different ($P \leq 0.05$) Ab-Leaf WoV (abaxial leaf surface facing the medium without mid vein); Ab-Leaf WV (abaxial leaf surface facing the medium with mid vein); Ad-Leaf WoV (adaxial leaf surface facing the medium without mid vein); Ad-Leaf WV (adaxial leaf surface facing the medium with mid vein); Stem-up (stem plated in upright position); Stem-in (stem plated in inverted position).

Of the 13 explant sources and treatments tested, significant differences were also observed in CRG, which measured the percentage of explant covered by callus after 30 days of *in vitro* culture (Table 3). Of the floral tissues, the calyx surface showed the highest CRG (70–96 %). Whereas for the vegetative tissues, the stem treatments (explants plated in either the upright or inverted positions) and the petiole gave the highest CRG. Stem explants plated in the upright position showed surface callus formation covering between 30.17 and 85.56 % of the tissue (similar to the stem-in inverted position), while the petiole showed a CRG of 42.33 and 71.67 %. It has been found that the explant source strongly influences the callus induction process. Previous reports have observed the same effect in other species, such as maize (Green and Phillips, 1975) and sugarcane (Guiderdoni and Demarly, 1988). Also, Declerck and Korban (1996) reported similar findings in peach, as did Ansley *et al.* (2000) for almond explants. In this study, explants consisting of leaves

without a midvein presented a higher percentage of callus development than leaves with midvein. It could be due to the fact that leaves without midvein have a greater surface area of wounded tissue, which is known to be more conducive to callus growth.

With regard to the culture media, taking into account all the treatments, WPM salts were the most successful in all the tested explants except for calyx and stem (inverted position) explants, for which, MS salts produced the higher callus induction rate (Table 3). Zhou *et al.* (2010) also found WPM to be more favorable for callus initiation than MS in a regeneration study in peach rootstocks. WPM-DK was the most successful treatment, inducing substantial callus growth in either of the light conditions tested; 64.58 % of the CRG in darkness and 38.78 % under a 16 h photoperiod. MS-DT was the second most prolific medium for callus induction, with 43.52 and 35.81 % CRG for 0 and 16 h light, respectively. Therefore, the results show that there is a combined effect induced by the culture medium and the plant growth regulators used.

The callus obtained from explants exposed to the 16 h photoperiod had a green and very compact texture in vegetative tissues (Figure 2A–C), whereas in petals (Figure 2F) and calyx (Figure 2G) the calli were mainly pink in color. The stamens (Figure 2E) turned pink, but no callus was formed. The appearance of calli in green tissues varied slightly depending on the explant type, but in general, it was composed of small green globular structures (Figure 2A, B). In young leaf explants (Figure 2A), the presence of callus was found mainly in the petiole or along the cut surface. These results agree with the previous research of Zhao *et al.* (2010) where young leaves of the hybrid *P. persica* x *Prunus davidiana* were tested. In this study, under 16 h light conditions, the callus that developed from buds and stems differed from leaves, being limited by the outer structure of the stems (Figure 2C) and buds, while the callus on leaf tissue was unconstrained and globular.

The appearance of all calli induced in the dark was the same, regardless of the explant type (Figure 2D) or the culture media. These calli were white to yellow, smooth, friable, and more voluminous than those obtained under light conditions. In all cultures, irrespective of exposure to light, the callus initially developed from cut tissue surfaces. Declerck and Korban (1996) tested different concentrations of auxins (2,4-D, dicamba) and cytokinins (BA, zeatin, kinetin, TDZ) in callus induction of peach. These authors maintained that in leaf tissues of *P. persica*, cytokinins are more likely to produce

Table 3. Influence of medium, photoperiod, and source of explant on callus relative growth (CRG) of six genotypes of peach

MEDIA	MS-DT			MS-DK			WPM-DT			WPM-DK			Average	
PHOTOPERIOD (Hours in light per 24h)	0	16	Average	0	16	Average	0	16	Average	0	16	Average	0	16
Ab-Leaf WV	32.06	15.64	**23.85**	10.81	11.58	**11.19**	15.89	13.08	14.49	61.31	27.11	**44.21**	**30.01b**	**16.85b**
Ab-LeafWoV	40.17	8.33	**24.25**	16.39	20.39	**18.39**	15.00	10.33	12.67	85.28	21.78	**53.53**	**39.21c**	**15.21b**
Ad-LeafWV	41.53	6.67	**24.10**	17.03	25.83	**21.43**	35.17	9.67	22.42	56.28	24.88	**40.58**	**37.50c**	**16.76b**
Ad-LeafWoV	38.72	24.22	**31.47**	31.39	26.83	**29.11**	45.00	22.33	33.67	74.89	36.92	**55.90**	**47.50e**	**27.58c**
Axillary bud	35.33	53.06	44.19	44.72	37.10	40.91	36.31	29.06	32.68	83.78	35.78	59.78	50.03f	38.75d
Calyx	80.83	88.00	84.42	**96.00**	91.50	93.75	78.50	72.17	75.33	85.83	**70.00**	77.92	**85.29j**	**80.42f**
Final bud	61.67	52.50	57.08	34.00	25.38	29.69	36.75	43.69	40.22	83.13	41.25	62.19	53.89g	40.71d
Juvenile leaf	29.33	19.00	24.17	23.96	13.61	18.78	43.47	20.29	31.88	52.50	43.33	47.92	37.32d	24.06c
Petals	22.50	26.50	24.50	17.67	23.50	20.58	19.00	21.67	20.33	41.67	31.67	36.67	25.21bc	25.83c
Petiole	52.67	**42.33**	47.50	60.67	60.50	60.58	48.33	55.33	51.83	**71.67**	57.50	64.58	**58.33h**	**53.92e**
Stamens	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	**0.00a**	**0.00a**
Stem-up	78.56	55.67	67.11	71.31	**30.17**	50.74	67.89	60.17	64.03	**85.56**	49.44	67.50	**75.83i**	**48.86e**
Stem-in	52.39	73.67	63.03	48.78	**25.17**	36.97	61.36	48.33	54.85	57.72	64.50	61.11	**55.06h**	**52.92e**
Total	**43.52**	**35.81**	**39.67c**	**36.36**	**30.12**	**33.24a**	**38.67**	**31.24**	**34.95b**	**64.58**	**38.78**	51.68d	45.78	33.99

CRG is the percentage of the explant covered by callus. Values in each column followed by the same letter are not significantly different ($P \leq 0.05$.) Ab-Leaf WV abaxial leaf surface facing the medium with midvein; Ab-Leaf WoV abaxial leaf surface facing the medium without midvein; Ad-Leaf WV adaxial leaf surface facing the medium with midvein; Ad-Leaf WoV adaxial leaf surface facing the medium without midvein; Stem-up Stem plated in upright position; Stem-in Stem plated in inverted position. Values in this row followed by the same letter are not significantly different ($P \leq 0.05$.)

chlorophyllous and compact callus cultures, whilst auxins increase callus production, inducing friable callus. To the best of our knowledge, those typologies are produced by the light since, in our study, the same media have been used in both conditions and the results were the same in the thirteen peach explants, genotypes, or treatments tested. Both typologies of peach callus we observed have been previously described as being embryogenic (white callus; Svircev *et al.*, 1993), or organogenic (green callus; Gentile *et al.*, 2002; Zhou *et al.*, 2010; Pérez-Jiménez *et al.*, 2012). Friable, white callus can be used effectively in cell suspension cultures due to its propensity to crumble (Bhansali *et al.*, 1991).

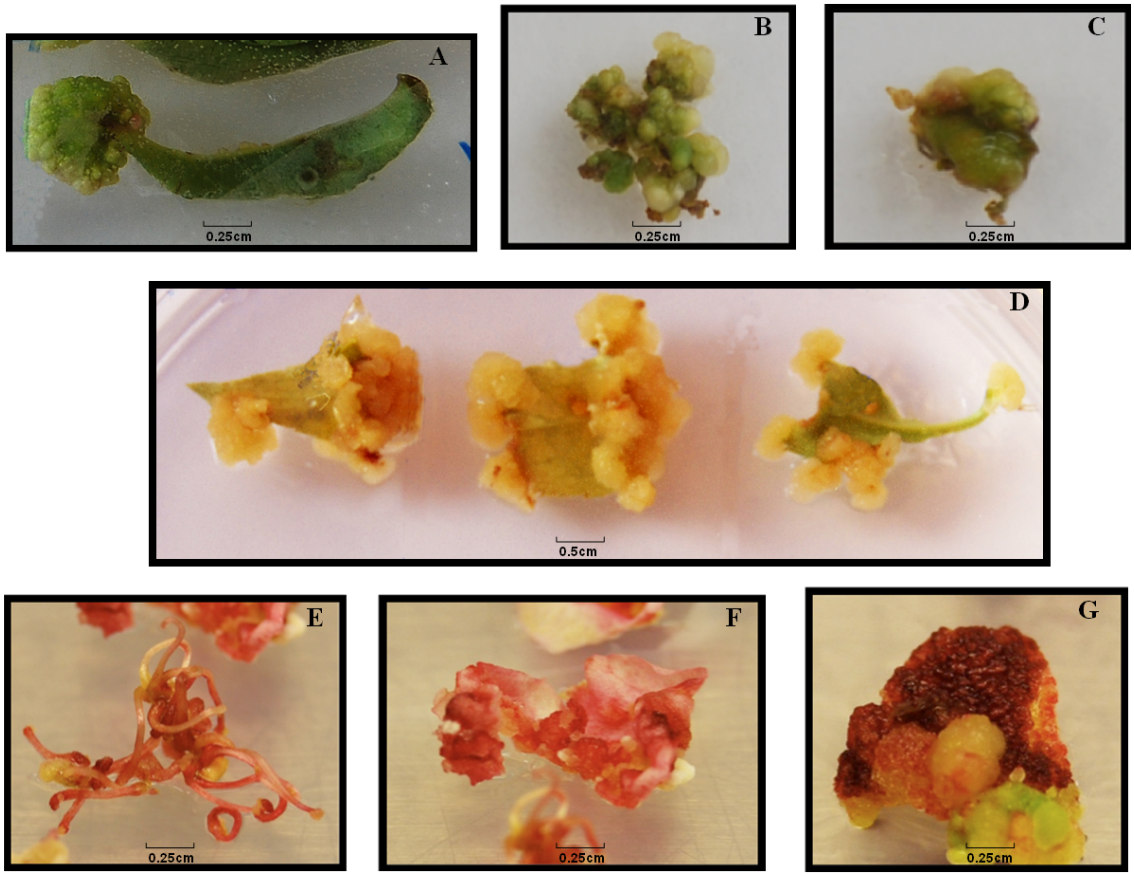

Figure 2. Differences between treatments and explants in callus induction of peach after 30 days. **(a)** Calli induced in a young leaf under a 16h light photoperiod. **(b)** Callus induced in a square piece of leaf without mid vein with the abaxial leaf surface facing the medium. **(c)** Callus induced in a stem explant plated in upright position under a 16h light photoperiod. **(d)** Calli induced in leaves with mid vein with the abaxial leaf surface facing the medium in darkness. **(e)** Stamens. **(f)** Callus induced in petals under a 16h light photoperiod. **(g)** Callus induced in calyx under a 16h light photoperiod.

In summary, this study demonstrates that the growth of *P. persica* calli is greatly influenced by the type of explant, the combination of plant growth regulators, culture media, and light conditions. The media composed of WPM supplemented with 2,4-D and KN induced a higher percentage of callus than the other media tested. A 16 h light photoperiod or constant darkness can be applied for callus induction for different purposes. White to yellow and friable callus was obtained under dark conditions and green compact, nodular callus was produced when explants were cultured in the light. The calyx was the most productive explant with regards to callus induction, followed by the vegetative explants, buds, stems, and petioles. No callus was obtained from the anthers or filaments with the conditions used in this study.

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CHAPTER III: SOMATIC REGENERATION

Results concerning this current chapter have been published as **Regeneration of Peach Cultivars (*Prunus persica* L. Batsch) and Peach Rootstocks (*Prunus persica* x *Prunus dulcis*) via organogenesis** (2012) *Plant Cell Tissue and Organ Culture*, 108:55-62 (see Annex I).

1 INTRODUCTION

Plant regeneration from adult tissues is the main obstacle to obtaining transgenic peach plants. A reliable protocol is required to generate non-chimeric transgenic plants (Pooler and Scorza, 1995). The development of a reliable regeneration system based on mature tissues is a prerequisite for application of transformation techniques for improvement of woody species (Litz and Grey, 1992; Liu and Pijut, 2008, 2010), and might be particularly useful to improve biotic and abiotic stress resistance and fruit quality (Srinivasan *et al.*, 2004). Plant regeneration is affected by many factors, such as genotype, culture medium, plant growth regulators, agar, type of explant and light conditions. For example, cytokinins are major factors in the induction of somatic organs (George, 1993; Magyar-Tabori *et al.*, 2010). The physiological and chronological age of explants and the *in vitro* culture period can influence organ formation (Hammerschlag *et al.*, 1985). In addition, the difficulty of regenerating plants from mature tissues of woody plants is well established (Smigocki and Hammerschlag, 1991).

Peach is one of the most recalcitrant species with regard to *in vitro* regeneration (Bhansali *et al.*, 1990; Padilla-Zakour *et al.*, 2006). Successful regeneration of peach plants is rare despite the use of juvenile explants as starting material. Immature seeds have been used most frequently as a vegetative explant in peaches (Meng *et al.*, 1981; Hammerschlag *et al.*, 1985; Scorza *et al.*, 1990; Bhansali *et al.*, 1991; Smigocki and Hammerschlag, 1991; Svircev *et al.*, 1993; Perez-Clemente *et al.*, 2004). Adventitious shoots have been regenerated successfully in peach from leaf explants excised from *in vitro* shoot apex cultures (Gentile *et al.*, 2002). Most authors (e.g., Declerck and Korban, 1996) have used segments of vegetative organs, such as leaves previously separated from the plant, for callus induction. Mezzeti *et al.* (2002) reported a similar protocol to that presented in this work for grape (*Vitis vinifera*), whereby callus is induced prior to excision of the explant from the plant.

This study describes an effective protocol for *in vitro* regeneration of peach via organogenesis. Three treatments comprising different concentrations of cytokinin and

auxin in the culture medium were compared. A histological examination of the organogenic calli were carried out in order to monitor the regeneration process and to confirm the neoformation of shoots.

2 MATERIAL AND METHODS

2.1 PLANT MATERIAL

Plant material was obtained from four-year-old peach trees grown at the Torreblanca experimental field station of the Instituto Murciano de Investigación y Desarrollo Agrario y Alimentario (IMIDA), Murcia, Spain. Nodal segments of the scion cultivars UFO-3®, Maruja®, Flariba® and Alice Bigi®, and of the peach × almond rootstocks Garnem® and GF677®, were collected and transferred to the tissue culture laboratory.

2.2 *IN VITRO* ESTABLISHMENT

The nodal segments were sterilized in a solution of 2 % (*v*/*v*) sodium hypochlorite and 0.1 % (*v*/*v*) Tween 20 for 2 h. Shoot cultures were established *in vitro* and subcultured monthly on M1 medium (Table 1) for three months. M1 medium was composed of Murashige and Skoog (MS) salts (Murashige and Skoog 1962), 3 % (*w*/*v*) sucrose and 0.7 % (*w*/*v*) of plant propagation agar (Pronadisa®). The pH was adjusted to 5.7 with 0.1 N KOH prior to autoclaving at 122 ºC (1.1 kg cm^{-2}) for 16 min. The proliferating shoots were cultured in climatic chambers at 25±1 ºC and with a 16 h light period (45 μmol m^{-2} s^{-1}; GRO-LUX, Sylvania).

2.3 CALLUS INDUCTION AND REGENERATION

Proliferation clusters were exposed to three treatments (Figure 1) consisting of different 6-benzyladenine (BA) and indolebutyric acid (IBA) concentrations and subculture cycles. Treatment 1 (T1) consisted of 30 days on M1; treatment 2 (T2) consisted of two cycles, 30 days on M1 and 30 days in on M2; and treatment 3 (T3) consisted of 30 days in on M1, 30 days in on M2 and 30 days in on M3. After treatment, the regeneration capacity was tested on OM medium for all treatments (Table 1 and Figure 1).

The organogenic calli were obtained from the base of proliferation clusters induced on M1 medium (Table 1). Prior to culturing on organogenic medium (OM), the calli were isolated and sliced. Sections were approximately 3 mm thick and were divided equally

between longitudinal and transversal cuts to rule out position effects on regeneration capacity.

2.4 ELONGATION AND ROOTING

Regenerated plants were separated and transferred to elongation medium (EM) to increase their survival capacity prior to acclimatization (Table 1). Explants approximately 5 cm long were transferred to rooting medium (RM) (Table 1), and acclimated when the roots were at least 2 ± 0.2 cm long.

Table 1. Composition of the culture media used during the induction (M1, M2, M3), regeneration (OM), elongation (EM) and rooting (RM) phases.

	M1	**M2**	**M3**	**OM**	**EM**	**RM**
Salts	MS	MS	MS	MS	MS	MS
NAA (mg l^{-1})	-	-	-	1	-	-
IBA (mg l^{-1})	0.1	0.1	0.1	-	-	1.5
BA (mg l^{-1})	1	1.5	2	2	-	-
Sucrose (g l^{-1})	30	30	30	30	30	30
Agar (g l^{-1})	7	7	7	7	7	7

MS = Murashige and Skoog salts; NAA = α-naphthalene acetic acid; IBA = indolebutyric acid; BA = 6-benzyladenine

2.5 EXPERIMENTAL DESIGN AND DATA COLLECTION

Data were recorded for 25 calli from each cytokinin concentration (total 75 calli). Treatments comprised cut orientation (longitudinal vs transversal) and cultivar. Data on shoot production (number of shoots per cluster) was recorded prior to sectioning. Other measured parameters were frequency of organogenic calli (FOC), organogenesis rate (OR) and percentage of callus with somatic shoots. Each experiment was repeated three times. All data were subjected to ANOVA and the significance ($P \leq 0.05$) of differences between mean values was tested using Duncan's new multiple range test.

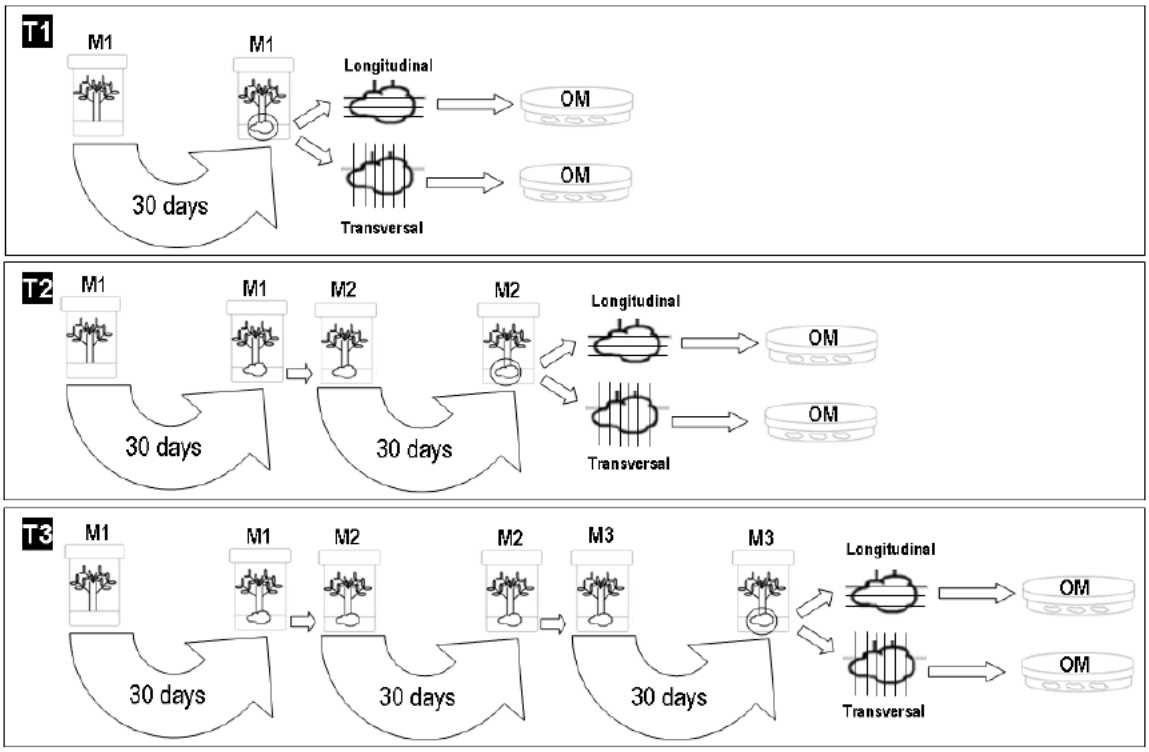

Figure 1. Treatments for callus induction and shoot regeneration. M1 = MS + 0.1 mg l^{-1} IBA + 1 mg l^{-1} BA. M2 = MS + 0.1 mg l^{-1} IBA + 1.5 mg l^{-1} BA. M3 = MS + 0.1 mg l^{-1} IBA + 2 mg l^{-1} BA. OM = MS + 1 mg l^{-1} ANA + 2 mg l^{-1} BA.

2.6 HISTOLOGY

Slides of the regenerating calli were prepared for histological examination. After culture for three months on OM medium, 15 calli were sampled to observe their tissue organization. The fixation procedure was based on that of Jensen (1962). The calli were stained with hematoxylin and eosin. Explants were dipped in FAA fixative (4 % (*v*/*v*) formaldehyde, 70 % (*v*/*v*) acetic acid and 70 % (*v*/*v*) alcohol; 1:1:18) for 24 h. The fixed material was dehydrated in a tertiary butyl alcohol (TBA-Merck®) series from 50 % to 100 %. The samples were transferred four times to liquid paraffin at 60 °C. After 24 h, the samples were embedded in paraffin blocks, and 10 µm thick sections were cut with a LEICA RM 2155 microtome and mounted on SuperFrost slides using TESPA glue. Hydration was carried out with xylol and decreasing concentrations of alcohol. The sections were stained with hematoxylin and eosin and dehydrated in xylol and increasing concentrations of alcohol. Samples were again hydrated using xylol and increasing concentrations of alcohol. Coverslips were mounted in DPX (Leica Microsystems), and the slides were observed under an Olympus SZX10 stereomicroscope and Olympus BH2-

RFCA microscope at 4×, 10× and 40× magnification. Motic Images Advanced 3.2 software was used to capture digital images.

3 RESULTS

3.1 CALLUS INDUCTION

Calli were induced from somatic cells during the first week after transfer to the induction media. The calli appeared at the stem base where the stem was in contact with the induction medium (Figure 2A). During the next two weeks, the calli grew in size, and became green and more compact. During the fourth week of culture, green compact nodules appeared on the surface of the calli.

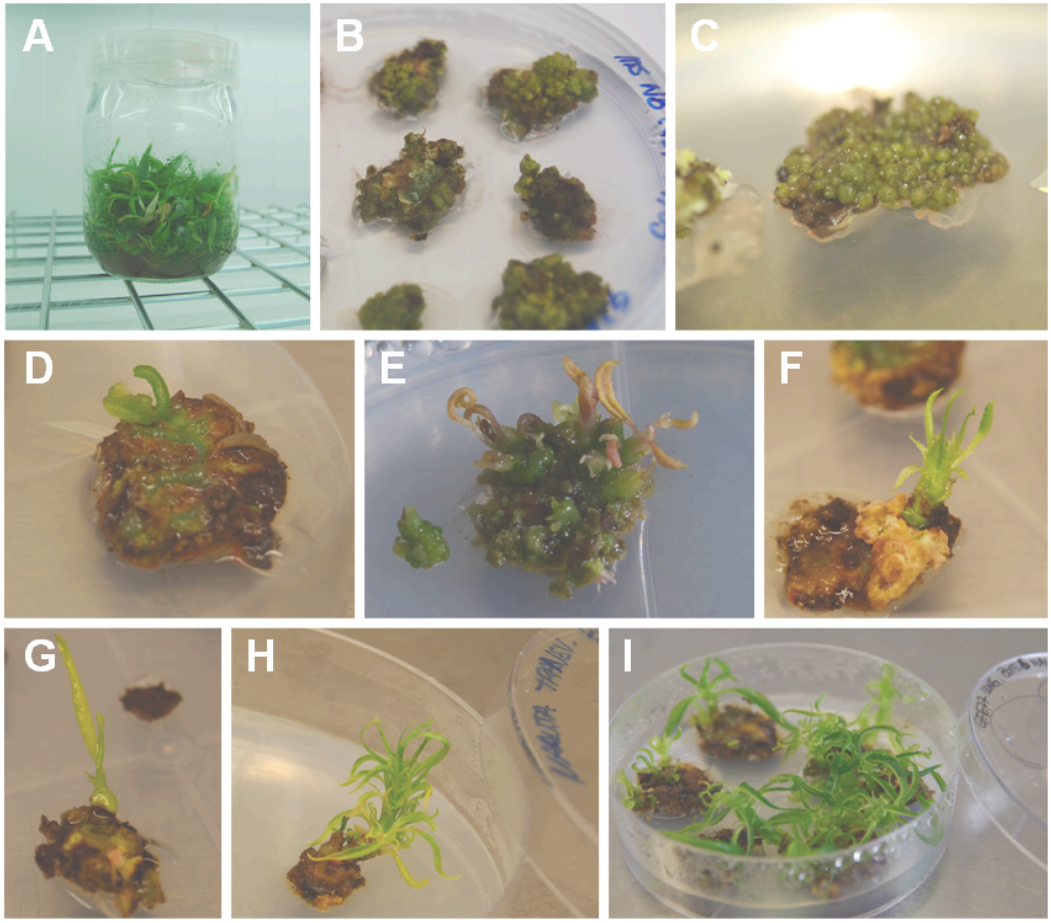

Figure 2. Adventitious shoot regeneration from *in vitro* nodal segment explants of peach cultivars and peach × almond hybrids. **(a)** Proliferation cluster of GF677® *in vitro*. **(b)** Slices of Garnem® callus in OM. **(c)** Callus formation on a slice of Garnem® in OM. **(d)** Adventitious shoot on Alice Bigi® callus. **(e)** Adventitious shoots on Garnem® callus. **(f)** Adventitious shoot on UFO-3® callus. **(g)** Adventitious shoot on Flariba® callus. **(h)** Adventitious shoot on Maruja® callus. (**i**) Adventitious shoots on GF677® calli.

3.2 ADVENTITIOUS SHOOT ORGANOGENESIS

After transfer of callus slices to OM, the calli turned darker green and nodule growth was stimulated. Initially, two zones in the slices could be differentiated: an internal white zone and an external zone covered by green nodules (Figure 2B, C). After culture on OM for one week, shoots started to develop from the nodules on the callus surface (Figure 2D–I), but some of the nodules gave rise to shoots after only the third or fourth week. Organogenesis began 10 days after transfer to OM and continued for up to 3 months.

3.3 CULTIVAR RESPONSE

Table 2. Frequency of organogenic callus (FOC) formation and maximum number of shoots produced per callus in peach cultivars and peach × almond hybrids.

Genotype	FOC (%)				Max. shoots number per callus
	T1	T2	T3	Mean	
Garnem®	84.0	91.6	66.6	80.8 a	10 a
GF677®	80.0	91.6	75.0	82.2 a	29 a
Flariba®	0	8.3	0	2.8 b	2 b
Maruja®	0	16.6	0	5.6 b	1 b
UFO-3®	0	25.0	0	8.3 b	2 b
Alice Bigi®	0	8.3	8.3	5.6 b	1 b
Mean	27.3 b	40.2 a	24.9 b		

Values in each column followed by the same letter are not significantly different ($P \leq 0.05$).

Differences between the cultivars were statistically significant ($P \leq 0.05$) and two groups were distinguishable, namely peach × almond hybrids and peach cultivars. The highest regeneration rates were obtained for the hybrids (Table 2). Most GF677® and Garnem® calli were organogenic and more than 80 % of the calli produced shoots; 29 and 10 shoots per callus, respectively, were the maximum number of new shoots obtained for the hybrids. In contrast, the peach cultivars produced no more than one or two shoots per callus in all of the treatments, and showed lower FOC values than the hybrids (Table 2).

Differences between cultivars were also evident in the proliferation rate (Figure 3). The highest frequency of organogenic calli were obtained from the base of the most proliferative callus clusters, as demonstrated by the low positive Pearson's correlation coefficient ($r = 0.463$).

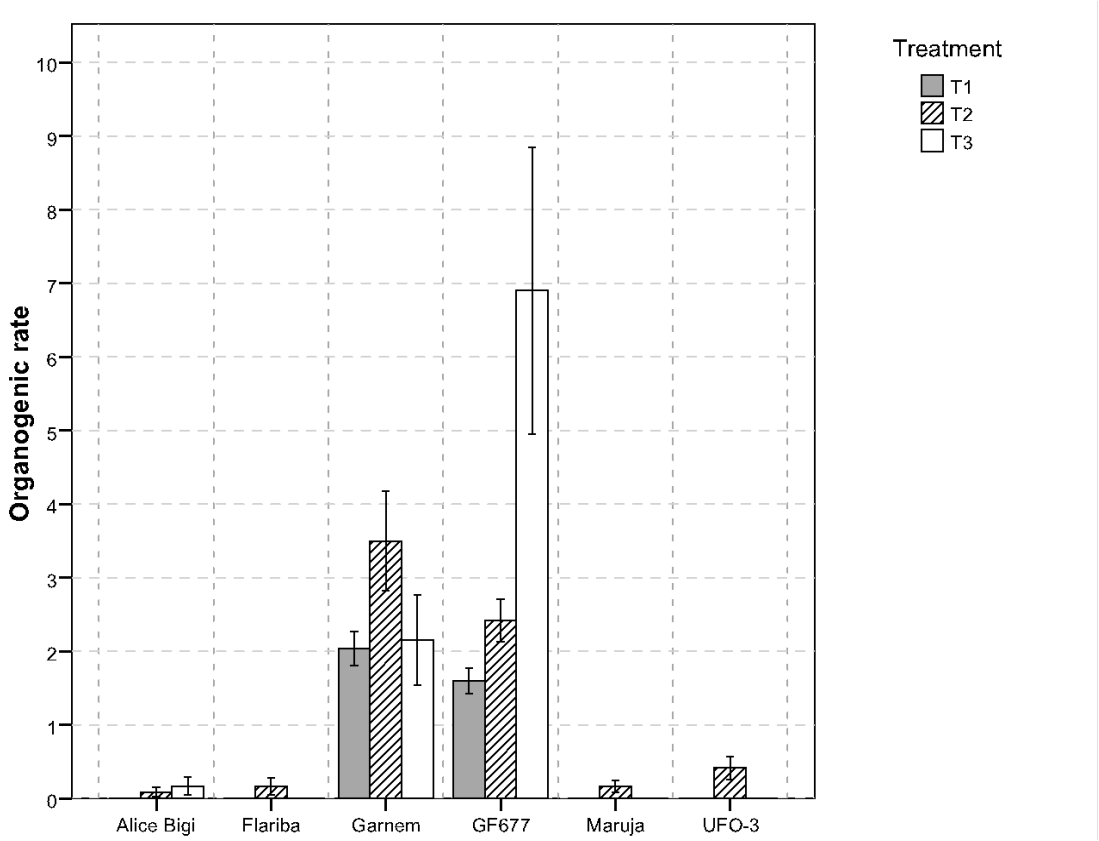

Figure 3. Genotype and treatment effects on organogenesis rates in peach cultivars and peach × almond hybrids. Vertical bars represent the standard deviation.

3.4 TREATMENT RESPONSE

The proliferation rate increased progressively from T1 to T3. The T3 treatment also showed the highest regeneration rate (Figure 4A). Regeneration was observed in calli from the clusters that showed the highest proliferation rates (Figure 4B). The T1 treatment induced organogenesis in the hybrids but did not induce shoot regeneration in any of the peach cultivars (Figure 3). T2 induced organogenesis in all of the genotypes and yielded the highest organogenesis rate. The differences between the treatments were significant

($P \leq 0.05$). Treatments T2 and T3 induced identical numbers of shoots in Alice Bigi® (Table 2). T3 showed the lowest mean regeneration rate for five of the six genotypes.

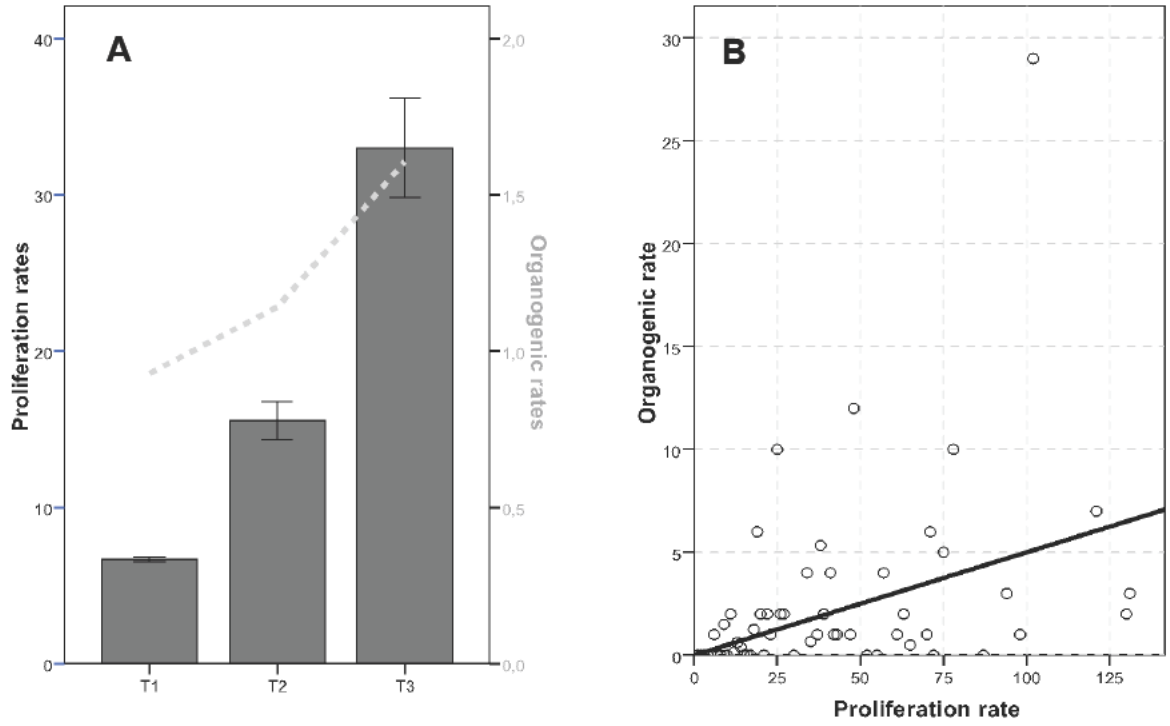

Figure 4. Relationship between number of shoots obtained per cluster (proliferation rate) and the percentage of callus with somatic shoots (organogenesis rate). **(a)** Histogram of organogenesis rates in each treatment. The dotted line represents the proliferation rate. **(b)** Regression of organogenesis rate and proliferation rate.

3.5 EFFECTS OF CUT ORIENTATION

The mean regeneration rates obtained for longitudinal and transversal explant sections did not differ significantly in any of the treatments or cultivars (Table 3). Nevertheless, transversal explants of UFO-3® and Garnem® on T3 showed slightly higher regeneration rates than longitudinal sections. In addition, the morphogenic capability of the shoots did not differ significantly (Figure 5).

Table 3. Effect of culture medium and explant cut orientation on frequency of organogenic callus (FOC) formation in peach cultivars and peach × almond hybrids.

Genotype	Treatment	FOC (%)	
		Longitudinal	Transversal
Garnem®	T1	75.2a	92.1a
	T2	100a	80.5a
	T3	50.3b	83.4a
	Mean	75.2a	85.3a
GF677®	T1	75.2a	85.5a
	T2	100a	83.1a
	T3	67.6a	83.1a
	Mean	80.9a	83.9a
Flariba®	T1	0a	0a
	T2	17.1a	0a
	T3	17.1a	0a
	Mean	11.4a	0a
Maruja®	T1	0a	0a
	T2	17.2a	17.1a
	T3	17.2a	17.1a
	Mean	11.5a	17.1a
UFO-3®	T1	0a	0a
	T2	0b	50.2a
	T3	0b	50.4a
	Mean	0b	50.2a
Alice Bigi®	T1	0a	0a
	T2	0a	17.2a
	T3	0a	17.2a
	Mean	0a	17.2a

Values in the same column followed by the same letter are not significantly different ($P \leq 0.05$).

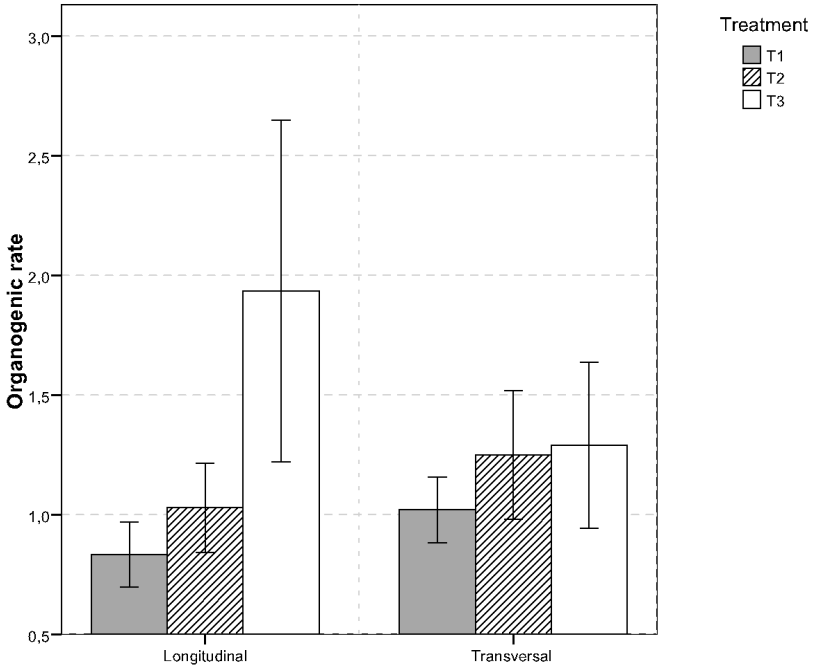

Figure 5. Effect of cut orientation on regeneration rates in the different treatments. Vertical bars represent the standard deviation.

3.6 HISTOLOGICAL OBSERVATIONS

The picture sequence in Figure 6 was taken under the stereomicroscope or microscope. Note the different structures involved in somatic plant development. During callus formation, undifferentiated cells were produced from the stem base. Hence, no organized tissue is discernible in Figure 6A. The callus formed did not preserve any feature of the stem cells, in case a bud was present at the base of the stem. In Figure 6A, the callus (ca) produced two shoots (sh). A cross section of one of the shoots can be observed in the bottom of the picture on the left, while a longitudinal section of the main shoot can be observed in the upper part of the picture, also on the left. On the right hand side of the second shoot, a meristemoid (me) can be observed and a leaf primordium on its right. The differentiating shoot (Figure 6B) comprised an external cell layer, the tunica, and an internal mass of cells, the corpus. The meristemoid (Figure 6C) comprised a group of meristematics cells that are smaller than the typical callus cells and contain a large central nucleus, which increased the nucleus: cytoplasm ratio of the cell. The external cells form the tunica and the remaining cells give rise to the corpus. Several leaf primordia can be seen growing from the s surface of the callus in Figure 6D.

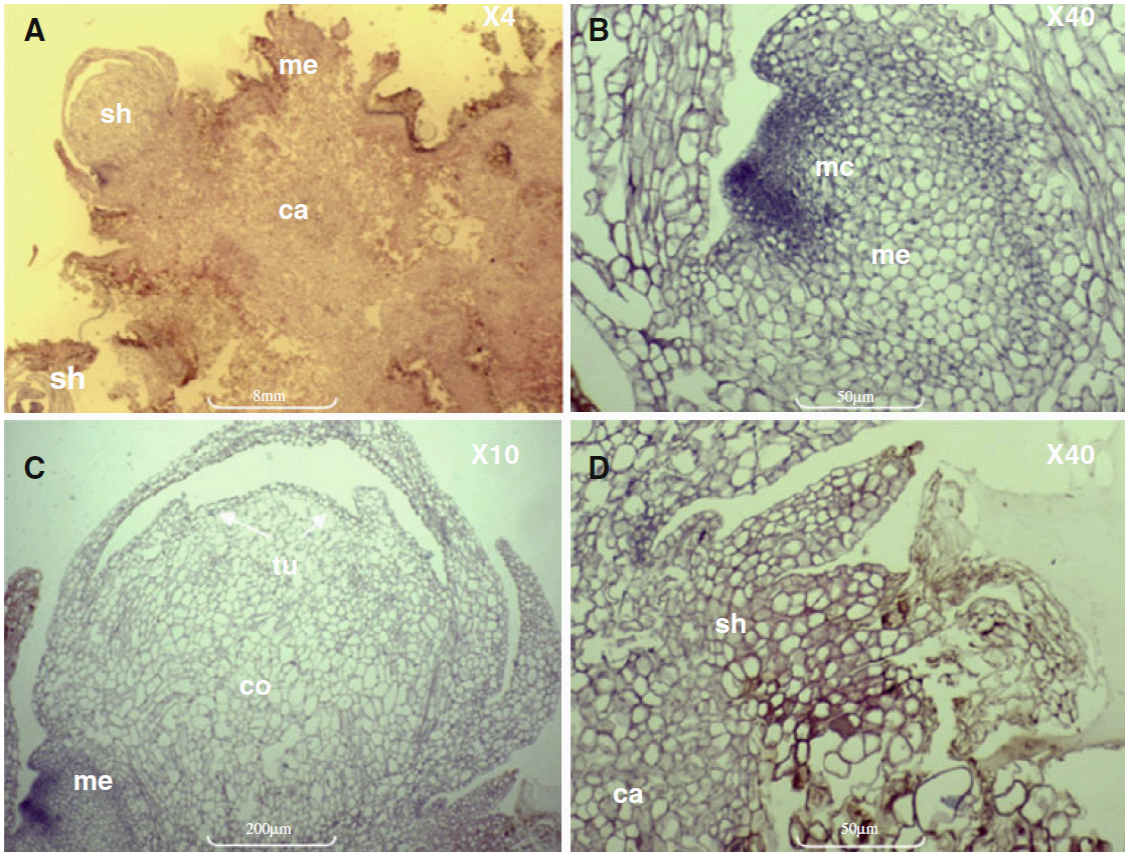

Figure 6. Histological sections of organogenic callus and adventitious shoots of the peach × almond hybrid rootstock Garnem®. **(a)** Transverse section of the callus. **(b)** View of the developing meristemoid. **(c)** Longitudinal section of the emerging bud. **(d)** Leaves and a new shoot emerging from the callus. ca callus, co corpus, mc meristematic cells, me meristemoid, sh shoot, tu tunica.

4 DISCUSSION

This study describes an effective protocol for the *in vitro* generation of somatic peach plants via organogenesis, The procedure is essential for the genetic transformation of the species and enables one of the major barriers hindering peach genetic improvement programs to be overcome. Although peach transformation techniques have been perfected, an effective regeneration protocol was still lacking. Previous studies of *in vitro* regeneration of peach carried out by other research groups used material derived from seeds, which is of scientific but not agronomic utility. To our knowledge, the only previous study that successfully regenerated peach plants using adult tissues is that of Gentile *et al.* (2002), but reproduction of these authors' results has proved difficult.

Callus development is the critical stage in the regeneration process. The protocol described here to obtain shoots by organogenesis utilized calli that developed at the base of

the proliferation cluster. New calli developed around the cut edges of the sections, as previously reported (Declerck and Korban, 1996). The callus induction process was initiated at the base of the stem in the cut zone where the cells were in contact with the culture medium and plant growth regulators. Callus induction then extended to the rest of the basal side of the stem, producing a large clump of callus below the explant. Green, compact callus with nodules on its surface formed, as described by Gentile *et al.* (2002). The organogenic callus developed as a result of the gradually increasing cytokinin content (Mezzetti *et al.*, 2002) and increasing endogenous hormone concentration produced by the plant while it was growing. Endogenous and exogenous regulators induce somatic shoot formation.

In this type of organogenesis system, new shoots are formed following suitable hormonal treatment and differentiate in three organogenesis stages: competence (Howel *et al.*, 2003), determination (Gahan *et al.*, 2008) and morphogenesis (Sugiyama, 1999). This type of protocol, involving proliferation clusters, has been successfully applied to other plant species such as *Vitis vinifera* (Mezzeti *et al.*, 2002). Of the different cytokinin treatments applied, T2 yielded the highest organogenic callus frequency and number of shoots per explant. This treatment consisted of two consecutive culture cycles with an increasing concentration of BA, which has been used as an organogenic regulator in several species, for example, in sour and sweet cherry (Tang *et al.*, 2002), pistachio (Tilkat *et al.*, 2009), *Prunus persica* × *P. davidiana* (Zhou *et al.*, 2010), *Colocynthis citrullus* (Ntui *et al.*, 2009), and blackberry (Gupta and Mahalaxmi, 2008). The T2 treatment was successful for all of the peach cultivars and *P. persica* × *P. dulcis* hybrids studied, and provided a mean of 40.2 % organogenic calli. According to Svircev *et al.* (1993), peach regeneration rates depend on genotype. The fact that the two hybrids included in the present study showed a similarly high regeneration response to hormones as the peach cultivars might reflect the parentage of the hybrid genotypes. Peach × almond hybrids demonstrate hybrid vigor in general and both GF677® and Garnem® have been described as particularly vigorous genotypes (Felipe, 2009). Although almond is one of the most recalcitrant species for *in vitro* culture (Ainsley *et al.*, 2000), the two peach × almond hybrids showed the highest regeneration rates in the present study. Peach is highly inbred, and inbreeding depression (Charlesworth and Charlesworth, 1987) might be partially responsible for the low regeneration rates observed.

The differences in organogenesis between the four peach cultivars were not significant and the fact that they were typologically distinct (representing peach, pavia, nectarine and flat-peach types) did not affect their regenerative capability. However, this finding must be corroborated using additional cultivars. Similarly, the type of cut (longitudinal vs. transversal) did not affect the organogenic capability of the cultivars. Although transversal sections (except in T3) were slightly more effective than longitudinal sections, the difference was not statistically significant.

An important parameter was the relationship between the organogenesis rate and the proliferation rate of the clusters from which the explants arise, which indicates that it would be convenient to preselect the most proliferative clusters to obtain calli with higher proliferation rates. The endogenous levels of the hormones supplied by the plant appear to facilitate peach organogenesis. In previous studies the hormone levels have not been suitably adjusted, and it is advisable to further investigate endogenous hormones *in vitro* to identify which plant growth regulators the plant is supplying to the organogenic callus during callus formation by the explant.

With the above in mind, we wondered whether the regenerated calli might be preformed in the callus obtained. To clarify this possibility, histological examination of longitudinal and transversal sections was undertaken. In all cases, shoot neoformation took place in the external part of the callus, where no vascular connections with the stem are present, demonstrating that axillary buds are not embedded in the callus. Moreover, given the large number of shoots that differentiated from some calli (up to 29 per callus) (Table 2), it is impossible for the shoots to be preformed at the base of the stem. The histological examinations were helpful to closely observe organ development. According to Amoros *et al.* (1991), cellular differentiation results in the typical structure of the differentiating shoot, which comprises the tunica and corpus. Meristematic cells were more intensely stained due to the process of multiplication. Growth tissue with a high DNA content, as well as the small size of the cells, ensure that the hematoxylin is more deeply fixed, giving it a more intense colour. As Ghimire *et al.* (2010) described, shoots differentiate from cells on or near the callus surface, and those cells in closest contact with the culture media.

The T2 treatment was the most efficient protocol for regenerating *Prunus persica* × *P. dulcis* hybrids and *P. persica* cultivars in the present study. It will be necessary to increase the regeneration rate if efficient peach transformation protocols are to be developed, which would ensure a high frequency of regeneration of transgenic genotypes. The protocol described herein should allow the development of improved transformation protocols for peach and other *Prunus* species.

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CHAPTER IV: ENDOGENOUS HORMONE CONTENT

PART I: COTILEDONS

1 INTRODUCTION

The establishment of an efficient *in vitro* somatic embryogenesis protocol for peach (*Prunus persica* L. Batsch) is required for application of transformation techniques for practical reasons and basic research. Peach is one of the most recalcitrant species with regard to *in vitro* regeneration (Bhansali *et al.*, 1990; Padilla *et al.*, 2006). Although plant regeneration through somatic embryogenesis has been achieved from immature cotyledons of peach by many authors (Meng and Zhou, 1981; Hammerschlag *et al.*, 1985; Scorza *et al.*, 1990; Bhansali *et al.*, 1991; Smigocki *et al.*, 1991; Svircev *et al.*, 1993; Pérez Clemente *et al.*, 2004), the routine success of the main protocols described has proven to be difficult.

Embryogenesis is affected by many factors: genotype, culture medium, plant growth regulators, gelling agent, type of explant, stress and light. However, of all these factors, plant growth regulators appear to play the most crucial role in somatic embryogenesis (Ruduś *et al.*, 2006). Hence, efforts must be made to obtain a deeper understanding of endogenous hormones as inducers of the embryogenic (E) potential of the explants. Indole-3-acetic acid (IAA), and two major active cytokinins, zeatin (Z) and zeatin riboside (ZR), are well known as determinant plant hormones in the E process (George, 1993b).

Plants cultured *in vitro* are exposed to high stress as they are placed on artificial conditions. It is thought that somatic embryogenesis could be a response to stress (Dudits *et al.*, 1995). Different molecules play regulatory roles in stress signalling, including those with hormonal activity such as jasmonic acid (JA) and abscisic acid (ABA) (Ruduś *et al.*, 2009). ABA is an important factor of *in vivo* and *in vitro* embryo maturation (Reidiboym-Talleux *et al.*, 1999) and an important hormone for initiation of E cultures (Jiménez, 2005). On the other hand endogenous ethylene is suggested to be involved in the regulation of embryogenesis *in vitro* (Kępczyńska *et al.*, 2009). In higher plants ethylene is synthesized from 1-aminocyclopropane-1-carboxylic-acid (ACC), while salicylic acid (SA) is an inhibitor of ethylene formation from ACC.

The aim of the present work is to evaluate whether differential endogenous hormonal compositions of E and non-embryogenic (NE) cotyledons could help to explain

their different capacity to induce somatic embryogenesis. The results would be useful for setting a reliable protocol of somatic embryogenesis induction in peach.

2 MATERIALS AND METHODS

2.1 PLANT MATERIAL AND CULTURE CONDITIONS

Open-pollinated fruit was collected from 8-years-old peach trees (cv. ZiseMay®) grown at the Torreblanca experimental field station of the Instituto Murciano de Investigación y Desarrollo Agrario y Alimentario (IMIDA), Murcia (Spain). The trees were watered daily using a drip irrigation system with 700 m^3 ha^{-1} per month at the moment of sampling. Fruit was harvested in April 2011, 50 days after full bloom, and stored overnight at 0 ± 2 ºC in a cold room.

Seeds were first removed from the endocarp and surface sterilized by agitating in a solution of sodium troclosene dehydrate (CTX-200/GR®; CTX S.A.U., Barcelona, Spain) at the concentration of 3.5 g l^{-1} containing 0.1 % (*v/v*) Tween-20 for 2 h in a laminar flow hood. After disinfection seed coats were removed and seeds were sliced in longitudinal cuts. The sections were placed in culture media composed of woody plant medium (Lloyd and McCown, 1980) (WPM), 0.7 % (*w/v*), plant propagation agar (Pronadisa®), 2.5 g l^{-1} of activated charcoal and 30 g l^{-1} of sucrose in sterilized plates, one sliced cotyledon per plate. The pH was adjusted at 5.7 with KOH (0.1 N) prior to autoclaving for 16 minutes at 1.1 kg cm^{-2} (122 ºC). The plates were cultured in climatic chambers at 25 ± 1 ºC and with a 16 h light period (45 μmol m^{-2} s^{-1}; Sylvania GRO-LUX fluorescent tubes).

After three *in vitro* subcultures for 30 days on the indicated medium, embryos started to develop from the cotyledons surface. Thereafter, cotyledons were divided in two different lines varying in their E capacity: NE and E cotyledons, and then were analysed.

2.2 HORMONE EXTRACTION AND ANALYSIS

The ABA, Cks [zeatin (Z) and zeatin riboside (ZR)], IAA, JA, SA and ACC were extracted and purified according to the method described by Dobrev and Kaminek (2002), and were analysed as described previously by Albacete *et al.* (2008). In summary, sampling material (1 g FW) was homogenized in liquid nitrogen and dropped in 2.5 ml of cold (-20 °C) extraction solution of methanol/water (80/20, *v/v*). Extracts were centrifuged at

20,000 *g* for 15 min at 4 °C, and the pellets were re-extracted for 30 min in an additional 2.5 ml of the same extraction solution. Supernatants were collected and filtered through Sep-Pak Plus C18 (Waters,Milford, MA, USA) to remove interfering lipids and pigments, and evaporated at 40 °C under vacuum. Residues were dissolved in 1 ml methanol/water (20/80, *v*/*v*) solution using an ultrasonic bath. The dissolved samples were filtered through 13 mm diameter nylon membrane Millex filters (Ø 0.22 μm) (Millipore, Bedford, MA, USA) and placed into tubes adjusting the volume to 1.5 ml with the extraction solution.

Analyses were carried out with an HPLC/MS system consisting of an Agilent 1100 Series HPLC (Agilent Technologies, Santa Clara, CA, USA) equipped with an autosampler connected to an Agilent Ion Trap XCT Plus mass spectrometer (©Agilent Technologies) using an electrospray interface. Before injection, 100 μl of each fraction was again filtered through Millex filters (Ø 0.22 μm). Each sample (8 μl) previously dissolved in mobile phase A was injected onto a Zorbax SB-C18 HPLC column (5 μm, 150 × 0.5 mm, Agilent Technologies) at 40 °C and eluted at a flow rate of 10 μl min^{-1}. Mobile phase A [water/acetonitrile/formic acid (94.9/5/0.1, *v*/*v*/*v*)] and mobile phase B [water/acetonitrile/formic acid (10/89.9/0.1, *v*/*v*/*v*)] were used for the chromatographic separation. The elution consisted of maintaining 100 % A for a period of 5 min, and then a 10-min linear gradient from 0 to 6 % B, followed by another 5 min linear gradient from 6 to 100 % B, and finally 100 % B kept for another 5 min. The column was equilibrated with the starting composition of the mobile phase for 30 min before each analysis. The UV chromatograms were recorded at 280 nm with the diode array detector module (Agilent Technologies). Different control samples with known concentrations of each component (0.001, 0.01, 0.05, 0.1, 0.2 and 0.5 mg l^{-1}) were also analysed under the same conditions. The mass spectrometer was operated in the positive mode with a capillary spray voltage of 3,500 V and a scan speed of 22,000 (m/z)/s from 50 to 500 m/z. The nebulizer gas (He) pressure was set to 30 psi, while the drying gas was set to a flow of 6 l min^{-1} at 350 °C. Mass spectra were obtained using the DataAnalysis program for LC/MSD Trap Version 3.2 (Bruker Daltonik, GmbH, Germany). For quantification of ABA and JA, calibration curves were constructed for each component analysed using internal standards: [$^2H_6$]*cis,trans*-abscisic acid and [$^2H_5$](±)-JA (Olchemin Ltd, Olomouc) (0.001, 0.01, 0.05, 0.1, 0.2 and 0.5 mg l^{-1}) and corrected for 0.1 mg l^{-1}. The ACC and SA were quantified by the external standard method, using the same concentration of the product (Sigma-Aldrich

Inc., St Louis, MO, USA). Recoveries ranged between 92 and 95 %. Three biological replicas were quantified per sample.

2.2 EXPERIMENT DESIGN AND DATA COLLECTION

Ten samples of each E and NE cotyledons were analysed. Three replications and three explants per analytical sample were used for this experiment. Significance was determined by ANOVA and the significance ($P \leq 0.05$) of differences between mean values was tested by Duncan's new multiple range test.

3 RESULTS AND DISCUSSION

In the present work E and NE cotyledons of peach have been analysed concerning their E capacity. To the best of our knowledge this is the first report in which the endogenous hormones levels have been related with the E competence of explants from *Prunus* species. In the present study immature cotyledons of peach (*Prunus persica* cv. ZiseMay®) were used as explants and a low ABA concentration was obtained in both NE and E cotyledons (Figure 1). According to the study of Piaggesi *et al.* (1991) in peach seeds (cv. Springcrest), ABA accumulation is detected during late stages of development. Hence, a low concentration of ABA was expected in this experiment since immature seeds were used. In addition, somatic embryos in peach cotyledons were in the yellow-green stage at the time of hormone analysis (Figure 2) and a precocious germination was appreciated (Figure 2E), which could be induced by the low ABA content (Zimmerman, 1993; Prewein *et al.*, 2004).

Analysis of the ABA concentration in cotyledons did not show significant differences between NE and E cotyledons. Many authors have reported higher ABA levels in E callus lines when compared to NE ones, especially in monocots (Jiménez, 2005). Nevertheless, no differences (Centeno *et al.*, 1997; Jimenez and Bangerth, 2000) or higher ABA levels in NE callus (Etienne *et al.*, 1993) have been reported in woody species. With the above in mind, endogenous ABA concentration has been related with somatic embryogenesis, but occasionally, sensitivity to hormones might explain differences in response between tissues and genotypes in their capability to become E. Actually, the sensitivity of the tissues to a change in the hormones concentration is more important than the change in the concentration itself (Trewavas, 1981).

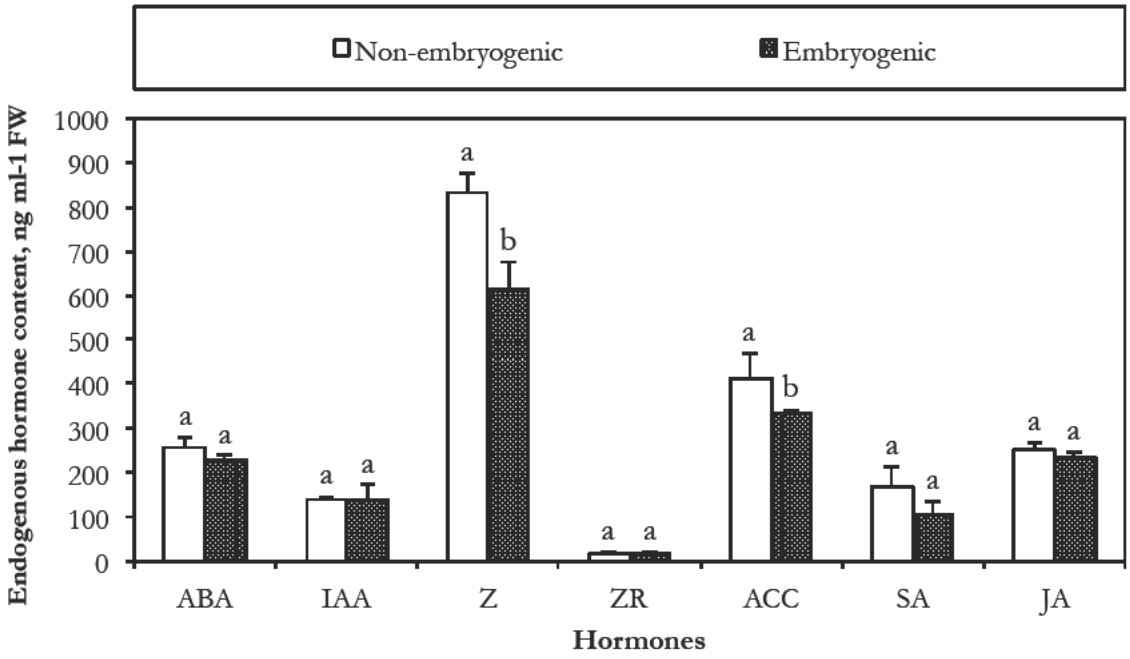

Figure. 1 Endogenous content in 1-aminocyclopropane-1-carboxylic-acid (ACC), salicylic acid (SA), indole-3-acetic acid (IAA), jasmonic acid (JA), zeatin (Z), abscisic acid (ABA) and zeatin (ZR) in embryogenic and non-embryogenic cotyledons of immature seeds of peach. Data represent average ± SD values. *Bars* with different lower case letters indicate a significant difference by LSD test ($P \leq 0.05$).

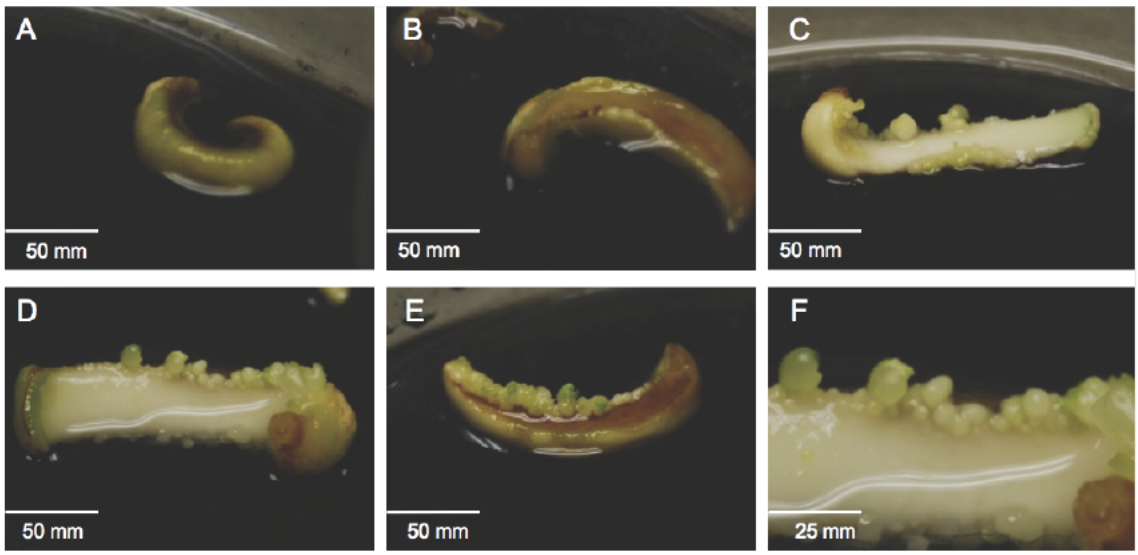

Figure 2. Somatic embryogenesis from immature cotyledons of peach inoculated on MS medium. **(a)** non embryogenic explant, **(b-f)** embryogenic explants.

Regarding to the results obtained in IAA levels, no differences between E and NE cotyledons were found (Figure 1). Auxins are considered to be the most important

hormones in regulating somatic embryogenesis (Srivastava, 2001), and endogenous IAA levels have been related with E competence. Notwithstanding, the role of IAA has been defined as a polar auxin gradient. Hence, the endogenous content in IAA increases to give the signal to induce the development of somatic embryos and decreases during the last phases on their growth and particularly during maturation (Jiménez, 2005). At the time of hormone analysis, cotyledons were expressing somatic embryos and some of them were already germinating (Figure 2E), therefore, the critical step where IAA levels are crucial has already passed.

Analysing the IAA/ABA ratio (Figure 3A) no differences were found between E and NE cotyledons according with the small differences observed in the levels of each individual phytohormone. Centeno *et al.* (1997) reported a ratio of E cotyledons of hazelnut (cv. Casina) nearly twice as high as that in the NE one. In contrast, our results pointed out that this ratio is not decisive in the capacity of the peach cotyledons in this study.

The results obtained analysing Z showed a higher content in NE cotyledons than in E cotyledons (Figure 1). This finding indicates a relationship between E capacity and endogenous content. This is supported by the results obtained by Centeno *et al.* (1997), where the authors found a lower Z concentration in E genotypes of hazelnut, and by Sáenz *et al.* (2010) in callus of coconut. Guiderdoni *et al.* (1995) reported lower levels of Z in E calluses than in the NE calluses and no difference in the ZR levels in sugarcane. These results are according with the ZR content obtained in this study (Figure 1). Lower content of ZR and Z has been related with more immature fruit (Centeno *et al.*, 1997). These results may support that most of the published somatic embryogenesis protocols in peach have been developed from immature seeds and cotyledons (Hammerschlag *et al.*, 1985; Bhansali *et al.*, 1990; Smigocki and Hammerschlag, 1991; Schneider *et al.*, 1992; Ye *et al.*, 1994; Pooler and Scorza, 1995; Pérez-Clemente *et al.*, 2004; Padilla *et al.*, 2006).

The Z/IAA ratio showed significant differences between E and NE cotyledons (Figure 3B). This ratio has been used as a physiological index of embryogenesis where a low Z/IAA ratio implies somatic embryo induction (George, 1993a). This hypothesis is supported by the results present in the current study where a low Z/IAA ratio is found in E cotyledons.

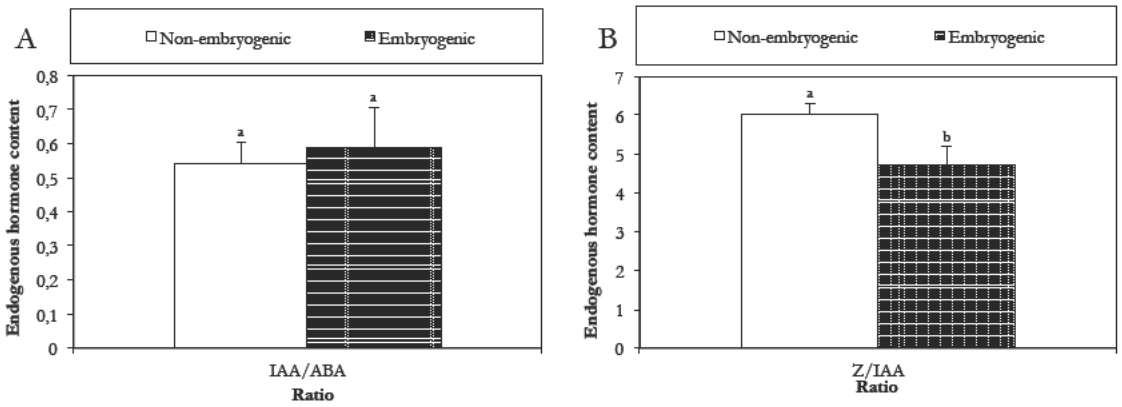

Figure 3. **(a)** Ratio between indole-3-acetic acid (IAA) and abscisic acid (ABA) in embryogenic and non-embryogenic immature cotyledons of peach. **(b)** Ratio between zeatin (Z) and indole-3-acetic acid (IAA) in embryogenic and non-embryogenic immature cotyledons of peach. Data represent average ± SD values. *Bars* with different lower case letters indicate a significant difference by LSD test ($P<0.05$).

NE cotyledons showed higher ACC content than E cotyledons (Figure 1). According to the review made by Buddendorf-Joosten and Woltering (1994), ethylene plays a negative role on induction of somatic embryogenesis. Huang *et al.* (2001) and Kępczyńska *et al.* (2009) reported a direct relation between concentrations of ethylene and ACC in E callus of alfalfa, and a low content of both in E callus. Regardless of the fact that SA is an inhibitor of the ethylene biosynthesis. The higher concentration was not found in E cotyledons; on the contrary, higher SA content was detected in NE cotyledons (Figure 1). There is controversy concerning the role of SA and ethylene. SA is an inhibitor of ethylene. However, SA has been reported as a stimulator of ethylene in peach (Molasiotis *et al.*, 2005) as well as in other species as carrot (Niessen, 1994) or potato (Liang *et al.*, 1997). It can be assumed that this is not only determinant for the data on the action of SA but also for the receptiveness of the cell to the compound.

The level of JA did not show any difference associated with E capacity (Figure 1). An increase of the stress-related phytohormones, ABA and JA, in somatic embryogenesis of *Medicago sativa* as a first signal of developmental changes (Ruduś *et al.*, 2009) has been published. On the contrary, in the present study no changes were found. JA and ABA probably increased their action after the slicing process 90 days before the analysis. Nevertheless, the levels could have decreased according to the adaptation of the cotyledons to the culture media.

In conclusion, these results are important since no external plant growth regulators were added to the media; therefore these results correspond to basal endogenous hormonal balance. Lakshmanan and Taji (2000) pointed out that detailed studies of those model systems in which addition of plant growth regulators are not necessary to induce somatic embryogenesis are very valuable to elucidate early regulatory events in embryo development. With the above in mind, Z shows a determinant role in the induction of direct somatic embryogenesis in cotyledons of peach as well as in the Z/IAA ratio; also in ACC changes in those cotyledons that induced somatic embryos. Hence, this study confirms the importance of analysing the hormonal balance of hormone in E tissues in peach where stress-related phytohormones (ABA, JA) do not seem to produce any direct effect related to somatic embryogenesis. However, further studies are required for unequivocal explanation of the complex mechanism that leads to somatic embryogenesis in peach.

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PART II: CALLI

1 INTRODUCTION

Peach (*Prunus persica* L. Batsch) is considered one of the major fruit species of the world. However, many biotic and abiotic stress factors affect plant growth and fruit quality. In the last decades, plant biotechnology has arisen as a viable option for the improvement of plants by means of plant tissue culture and genetic transformation. Notwithstanding, plant regeneration from adult tissues is the main obstacle for the obtention of transgenic peach plants (Pérez-Jiménez *et al*., 2012) due to the fact that peach is one of the most recalcitrant species with regard to *in vitro* regeneration (Bhansali *et al*., 1990; Padilla *et al*., 2006). Although several authors have published successful regeneration protocols, regeneration of peach plants is rare despite the use of seeds or cotyledons explants as starting material. The main disadvantage of developing a protocol from seed-derived material is that each genotype is unique and not a clone of the parent (Abbott *et al*., 2008). Only a few authors have developed somatic regeneration protocols using adult material (Gentile *et al*., 2002; Pérez-Jiménez *et al*., 2011).

The addition of plant growth regulators to the culture medium is the preferred way to induce *in vitro* morphogenic responses in most of the plant tissue culture systems evaluated (Jiménez, 2005). In organogenesis technology, 6-benzylaminopurine (BA) is widely used in woody species (Lakshmanan *et al*., 1997; Gentile *et al*., 2002; Arigita *et al*., 2005; Magyar-Tábori *et al*., 2010). However, plant growth regulators added exogenously exert part of their effect by modifying the concentrations of endogenous hormones (Gaspar *et al*., 1996) playing a major role in the regulation of morphogenesis (Fernando and Gamage, 2000). Endogenous growth factors are involved in the induction and determination of the morphogenic reactions and their concentrations will be spatially and temporally regulated in response to external culture conditions (Charrière and Hahne, 1998).

High levels of endogenous cytokinins and auxins have an important role in the initiation of proliferation centres in the explants (Valdés *et al*., 2001) and they are employed mostly to regulate cell division and differentiation in the explants. Indole-3-acetic acid (IAA) and two major active cytokinins, zeatin (Z) and zeatin riboside (ZR), are well known as determinant plant hormones in the morphogenic process (George, 1993).

It is acknowledged that *in vitro* culture is accompanied by stress conditions, as explants are transferred to synthetic environment and conditions. Jasmonic acid (JA) and abscisic acid (ABA) are stress-related phytohormones involved in the tolerance to environmental stress. Furthermore, it has been proved that ABA plays a regulatory role in organs induction (Rai *et al.*, 2011). On the other hand, the gaseous hormone ethylene, in addition to its role in fruit ripening, it also promotes the development and elongation of shoots. In fact, during the *in vitro* process sufficient ethylene can be accumulated in the culture flasks to regulate organogenesis and morphogenesis (George, 1993). In higher plants ethylene is synthesized from 1-aminocyclopropane-1-carboxylic-acid (ACC), while salicylic acid (SA) is an inhibitor of the ethylene formation from ACC.

Our previous studies established a reproducible organogenic protocol for peach cultivars and rootstocks (Pérez-Jiménez *et al.*, 2012). In the present study, our attempt is to establish a relationship between endogenous hormonal levels and *in vitro* morphogenic responses of different genotypes in peach and peach x almond hybrids. A first approach has been made since our results suggest that certain changes in the endogenous hormones are reflected in a differential morphogenic response, and these changes depend mainly on the genotype.

2 MATERIALS AND METHODS

2.1 PLANT MATERIAL

Plant material was obtained from 4-year-old peach trees grown at the Torreblanca experimental field station of the Instituto Murciano de Investigación y Desarrollo Agrario y Alimentario (IMIDA), located in Cartagena, Spain. Nodal segments of the scion cultivars UFO-3®, Flariba®, and Alice Bigi®, and of the peach x almond rootstocks Garnem® and GF677®, were collected and transferred to the tissue culture laboratory.

2.2 *IN VITRO* ESTABLISHMENT

The nodal segments were surface sterilized by agitating in a solution of sodium troclosene dehydrate (CTX-200/GR®; CTX S.A.U., Barcelona, Spain) at a concentration of 3.5 g l^{-1}, containing 0.1 % (*v*/*v*) Tween-20 for 2 h in a laminar flow hood. Shoot cultures were established *in vitro* and subcultured monthly on culture medium for 3 months. The medium was composed of Murashige and Skoog (MS) salts (Murashige and Skoog, 1962),

3 % (*w/v*) sucrose and 0.7 % (*w/v*) plant propagation agar (Pronadisa®) in 300 ml culture vessels each containing 100 ml culture medium. The pH was adjusted to 5.7 using 0.1 N KOH prior to autoclaving at 122 ºC (1.1 kg cm^{-2}) for 16 min. The proliferating shoots were cultured in climatic chambers at 25 ± 1 ºC and with a 16 h light period (45 μmol m^{-2} s^{-1}; Sylvania GRO-LUX fluorescent tubes).

2.3 CALLUS INDUCTION AND REGENERATION

The organogenic calli were obtained from the base of proliferation clusters induced as previously described by Pérez-Jiménez *et al.* (2012). Shoots were cultivated in culture media composed of MS salts, 3 % (*w/v*) sucrose and 0.7 % (*w/v*) plant propagation agar combined with 0.1 mg l^{-1} of indolebutyric acid (IBA) and 1 mg l^{-1} of BA (Duchefa®). After three *in vitro* subcultures for 30 days on the indicated medium, the callus was excised and transferred into organogenic medium. This medium was composed of MS salts, 2 mg l^{-1} of BA and 1 mg l^{-1} of naftalenacetic acid (NAA).

2.4 HORMONE EXTRACTION AND ANALYSIS

The ABA, cytokinins [Z and ZR], IAA, JA, SA and ACC were extracted and purified according to the method described by Dobrev and Kaminek (2002), and were analysed as described previously by Albacete *et al.* (2008). In summary, sampling material (1 g FW) was homogenized in liquid nitrogen and dropped in 2.5 mL of cold (-20 °C) extraction solution of methanol/water (80/20, *v/v*). Extracts were centrifuged at 20,000 *g* for 15 min at 4 °C, and the pellets were re-extracted for 30 min in an additional 2.5 ml of the same extraction solution. Supernatants were collected and filtered through Sep-Pak Plus C18 (Waters, Milford, MA, USA) to remove interfering lipids and pigments, and evaporated at 40 °C under vacuum. Residues were dissolved in 1 mL methanol/water (20/80, *v/v*) solution using an ultrasonic bath. The dissolved samples were filtered through 13 mm diameter nylon membrane Millex filters (Ø 0.22 μm) (Millipore, Bedford, MA, USA) and placed into tubes adjusting the volume to 1.5 mL with the extraction solution.

Analyses were carried out with an HPLC/MS system consisting of an Agilent 1100 Series HPLC (Agilent Technologies, Santa Clara, CA, USA) equipped with an autosampler connected to an Agilent Ion Trap XCT Plus mass spectrometer (©Agilent Technologies) using an electrospray interface. Before injection, 100 μl of each fraction was again filtered

through Millex filters (Ø 0.22 μm). Each sample (8 μL) previously dissolved in mobile phase A was injected onto a Zorbax SB-C18 HPLC column (5 μm, 150 × 0.5 mm, Agilent Technologies) at 40 °C and eluted at a flow rate of 10 μl min^{-1}. Mobile phase A [water/acetonitrile/formic acid (94.9/5/0.1, *v*/*v*/*v*)] and mobile phase B [water/acetonitrile/formic acid (10/89.9/0.1, *v*/*v*/*v*)] were used for the chromatographic separation. The elution consisted of maintaining 100 % A for a period of 5 min, and then a 10-min linear gradient from 0 to 6 % B, followed by another 5 min linear gradient from 6 to 100 % B, and finally 100 % B kept for another 5 min. The column was equilibrated with the starting composition of the mobile phase for 30 min before each analysis. The UV chromatograms were recorded at 280 nm with the diode array detector module (Agilent Technologies). Different control samples with known concentrations of each component (0.001, 0.01, 0.05, 0.1, 0.2 and 0.5 mg l^{-1}) were also analysed under the same conditions. The mass spectrometer was operated in the positive mode with a capillary spray voltage of 3,500 V and a scan speed of 22,000 (m/z)/s from 50 to 500 m/z. The nebulizer gas (He) pressure was set to 30 psi, while the drying gas was set to a flow of 6 l min^{-1} at 350 °C. Mass spectra were obtained using the DataAnalysis program for LC/MSD Trap Version 3.2 (Bruker Daltonik, GmbH, Germany). For quantification of ABA and JA, calibration curves were constructed for each component analysed using internal standards: [$^2H_6$]*cis,trans*-abscisic acid and [$^2H_5$](±)-JA (Olchemin Ltd, Olomouc) (0.001, 0.01, 0.05, 0.1, 0.2 and 0.5 mg l^{-1}) and corrected for 0.1 mg l^{-1}. The ACC and SA were quantified by the external standard method, using the same concentration of the product (Sigma-Aldrich Inc., St Louis, MO, USA). Recoveries ranged between 92 and 95 %. Three biological replicas were quantified per sample.

2.5 CYTOKININ IMMUNOCYTOCHEMISTRY

Z immunolocalisation was performed according the procedure, without embedding, of Dewitte *et al.* (1999), but with some modifications, using antibodies raised against ZR. Cross-reactivity of the affinity- purified anti-ZR antibodies with closely related compounds has been checked extensively, and this method has proved to be highly reliable (Dewitte *et al.*, 1999): the anti-ZR antibodies cross-react significantly *in vitro* only with free Z, zeatin ribosides and zeatin N-glucosides, but not with the O-glucosides. Upon aldehyde fixation of plant material, only free Z, but not the ribosides or the N-glucosides, are linked to the cellular proteins (Sossountzov *et al.*, 1988; Dewitte *et al.*, 1999). The O-glucosides are

retained by the fixative but are not recognized by the antibodies. Consequently, the immunolabelling with anti-ZR antibodies corresponds only to free Z. The cross-reaction with BSA was null on aldehyde-fixed material. Calli were fixed in a 0.5 % (*v/v*) glutaraldehyde and 3 % (*w/v*) paraformaldehyde in PBS for 2.5 h at +4 ºC following a 10 min initial vacuum infiltration. Sections (50 μm) were cut with a vibratome (Vibratome 1000, Technical Products International, St Louis, MO, USA). Sections were not attached to slides for incubation but were placed in the wells of tissue culture plates, so that penetration of the antibodies occurred on both faces, allowing the chemicals to reach most of the cells. Sections were first collected in ice-cold PBS, then the floated sections were pre-incubated (three times, 10 min each) in blocking buffer (0.1 % fish gelatine *v/v*, 0.5 % BSA *w/v*, 1 % normal goat serum *v/v*, glycine 0.15 % in PBS) and then in a Tween solution in PBS for 15 min. They were then incubated with rabbit primary anti-ZR antibodies (OlChemIm Ltd, Olomuc, Czech Republic) at a dilution of 1 / 200 in blocking buffer at 4 ºC overnight, followed by 1 h at room temperature. After four washes (10 min each) in PBS, sections were incubated with the secondary antibody (IGg anti-rabbit conjugated with alkaline phosphatase; Molecular Probes, Eugene, OR, USA) diluted in PBS (1 / 250) for 3 h at room temperature. After four washes (10 min each) in PBS and two washes (5 min each) in 100 mm Tris buffer (pH 9.5) containing 2 mm $MgCl_2$, the sections were allowed to react in the presence of nitroblue tetrazolium (0.01 % NBT) and 5-bromo-4-chloro-3-idolyl phosphate p-toluidine salt (0.01 % BCIP) in Tris buffer. The enzymatic reaction was monitored by light microscopy then stopped by adding PBS / EDTA. Controls omitting the primary antibody were done for each stage. Two other controls were used less extensively but also confirmed the specificity of the immunolabelling: ice-cold MeOH extraction overnight prior to aldehyde fixation led to the absence of any signal, and saturating the primary antibodies with 10 nm ZR strongly reduced the labelling. Sections were mounted in a PBS and glycerol mixture (1:1), immediately observed under an Axioscop Zeiss light microscope and photographed.

2.6 EXPERIMENT DESIGN AND DATA COLLECTION

Three replications with 6 calli from each of the cultivars per replication were used. The calli weight and the number of regenerated shoots per callus were recorded. Significance was determined by ANOVA and the significance ($P \leq 0.05$) of differences between mean values was tested by Duncan's new multiple range test.

3 RESULTS AND DISCUSSION

This work focused on the study of the organogenic capacity of callus from three cultivars of peach and two peach x almond hybrids. Although a few authors have published regeneration protocols in *Prunus*, the endogenous hormone variations concerning this event are still uncovered. Differences in regeneration between the cultivars were statistically significant ($P \leq 0.05$) and two groups were distinguishable, namely peach x almond hybrids and peach cultivars. The amount of regenerated plants in callus induced from the hybrids was about 3.8 and 6.3 times greater than in callus induced from the peach cultivars (Table 1). This is supported by the results showed by Pérez-Jiménez *et al.* (2012), whose findings pointed out that the endogenous differences in the hormonal content between the cultivars and the rootstocks might be causing differences in the organogenic response. Genotypes with high regeneration obtained also heavier callus (Table 1). It could be due to a higher sensitivity of the tissues in the genotypes with a larger response to plant growth regulators.

Table 1. Adventitious shoot regeneration from callus explants of three peach cultivars and two peach rootstocks.

Cultivars	Regeneration	Callus weight (g)
Flariba®	2.14±1.03b	0.56±0.07d
Alice Bigi®	3.57±0.97b	1.12±0.02c
UFO-3®	2.71±0.08b	0.62±0.06d
GF677®	13.43±2.15a	1.45±0.16b
Garnem®	13.57±3.03a	2.20±0.30a

Values in the same raw followed by the same letter are not significantly different ($P \leq 0.05$).

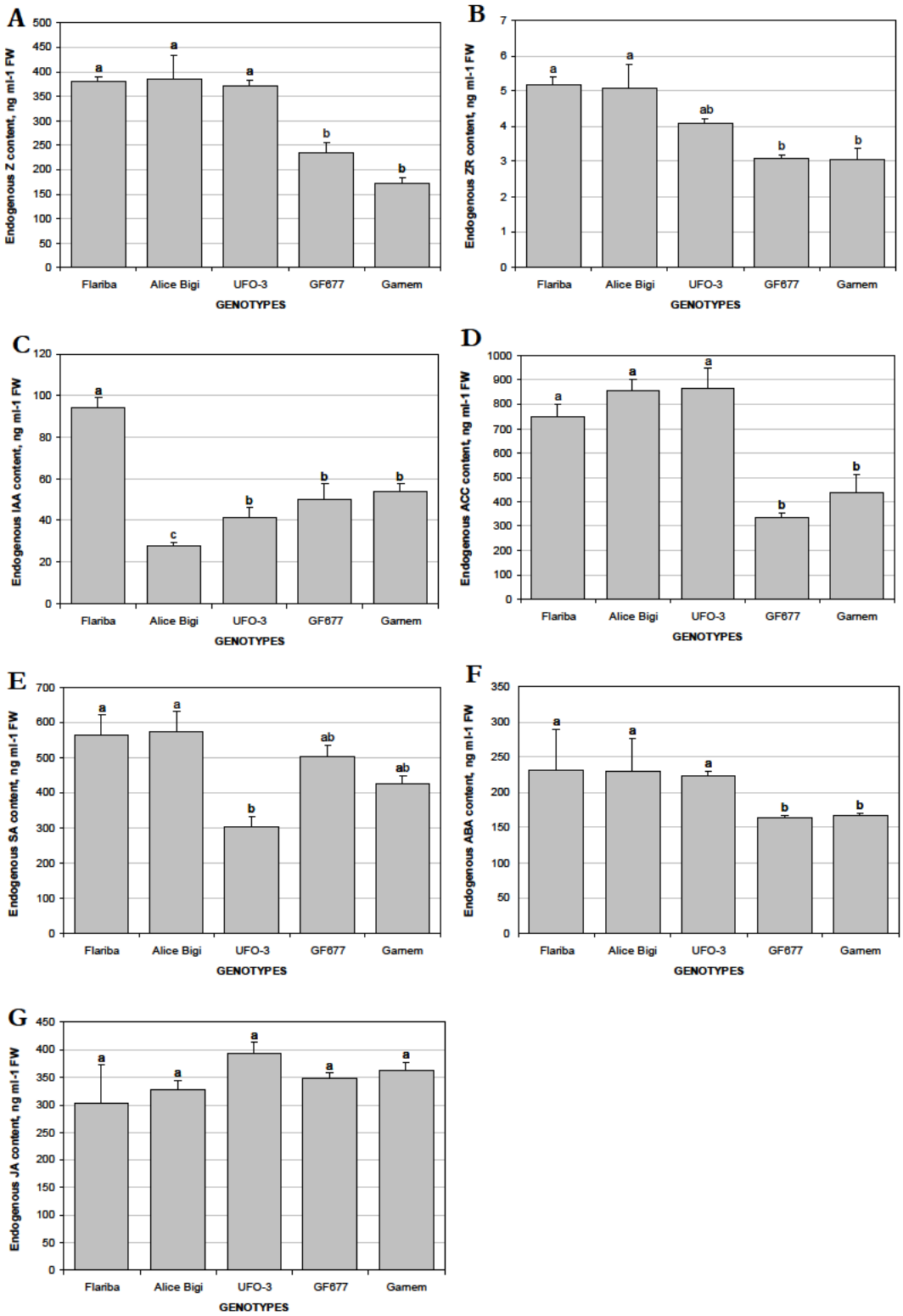

Figure 1. Endogenous content in **(a)** zeatin (Z), **(b)** zeatin riboside (ZR), **(c)** indole-3-acetic acid (IAA), **(d)** 1-aminocyclopropane-1-carboxylic-acid (ACC), **(e)** salicylic acid (SA), **(f)** abscisic acid (ABA) and **(g)** jasmonic acid (JA) in callus of peach (cv. Flariba®, cv. Alice Bigi®, cv. UFO-3®, cv. GF677®, cv. Garnem®). Data represent average ± SD values. *Bars* with different lower case letters indicate a significant difference by LSD test ($P \leq 0.05$).

The analysis resulting from the quantification of the two endogenous isoprenoid cytokinins (Z and ZR) in callus varied among the genotypes (Figure 1A-B). Z and ZR concentrations were higher in the callus of the peach cultivars than in the callus of the hybrids. These results were unexpected, since cytokinins have been linked to somatic organogenesis in tissue culture (George, 1993). Accordingly, high levels of Z were found in different organogenic explants of other woody plants (Centeno *et al.*, 1995; Valdés *et al.*, 2001; Malá *et al.*, 2005) when compared to non-organogenic ones, in the presence of BA.

Nevertheless, distinct results have been reported for ZR, from an increment in the more reactive genotypes (Valdés *et al.*, 2001), to the same response (Malá *et al.*, 2005) and a decrease in the amount (Centeno *et al.*, 1995) of organogenic explants. The differences in the morphogenic responses could be the result of differences in absorption and metabolism of BA (Arigita *et al.*, 2003), since explant tissues consist of cells with distinct capacity to respond to an induction treatment (Jiménez, 2005). The genotype plays also a major role in the differences among species, and in the least responsive species, such as woody plants, it is common to find genotypes that react more readily than others to a particular set of inductive conditions (Jiménez, 2005).

Immunolocalisation of Z was performed, using a light microscope (Figure 2), in calli of the five genotypes, in order to find any difference in the distribution at the histological level. At tissue level, immunoreactivity was detected in the entire surface of the callus in every genotype, with no differences amongst them. Labelling of Z was only absent from the conducts produced by tracheids in Flariba® and UFO-3®. The control sections, with the primary antibody omitted, showed no purple signals (Figure 2 A, C, E, G, I).

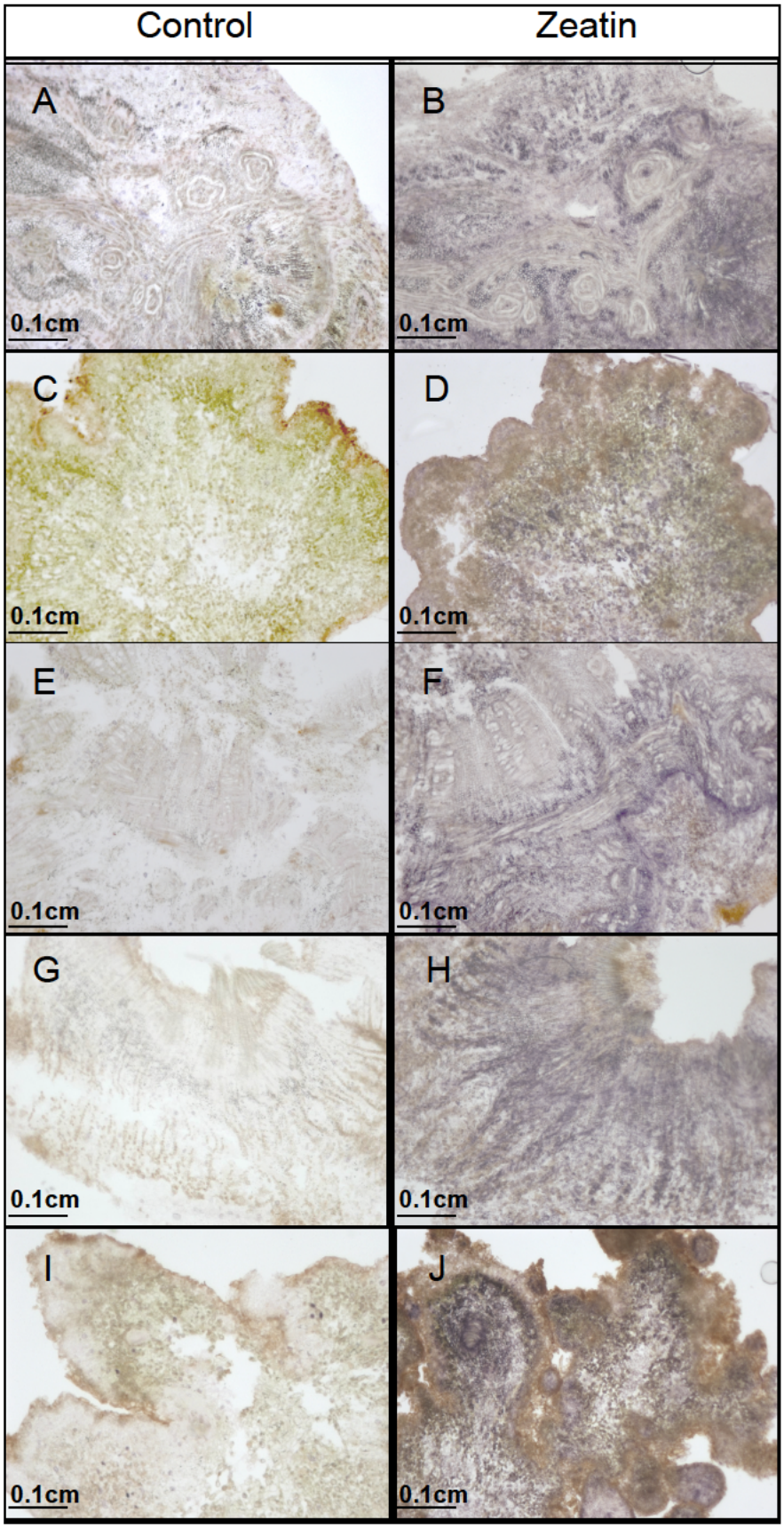

Figure 2. Zeatin immunolocalisation in transversal sections of callus collected from the basal part of shoots of **(a-b)** Flariba®, **(c-d)** Alice Bigi®, **(e-f)** UFO-3®, **(g-h)** GF677®, **(i-j)** Garnem®.

The endogenous IAA measured for the callus explants of different peach cultivars and rootstocks were presented in Figure 1C. IAA content in Flariba® more than doubled that of the rest of the studied genotypes. It has been previously reported that levels of IAA increase in explants showing somatic organogenesis (Malá *et al.*, 2005; Huang *et al.*, 2012). In addition, IAA has been related to proliferation and maintenance of callus (Centeno *et al.*, 1996). In this study, no relation was found between IAA and callus weight or regeneration. But, occasionally, sensitivity to hormones might explain differences between tissues and genotypes in their capability to become organs. Actually, the sensitivity of the tissues to a change in the hormones concentration is more important than the change in the concentration itself (Trewavas, 1981). Nevertheless, according to the results, the most important is that no general trend could be found that related IAA levels and shoot formation in peach, as it has been previously observed in pumpkin (Zhang *et al.*, 2008).

A significant effect was observed in the case of the IAA/ABA ratio (Figure 3A). The IAA/ABA content is markedly higher in Flariba® than in the rest of genotypes, due to the high IAA content detected in this cultivar. On the contrary, Alice Bigi® and UFO-3® showed the lowest content amongst the genotypes. In the most regenerative genotypes in this experiment, GF677® and Garnem®, medium IAA/ABA levels were obtained. However, analysing the Z/IAA ratio (Figure 3B) no relation was found associated to organogenesis. Z/IAA content was higher in Alice Bigi®, UFO-3® and lower in GF677®, Garnem® and Flariba®. IAA content in Flariba® is manifestly influencing the ratio, resulting in low Z/IAA levels reported as non-organogenic index in elm (Malá *et al.*, 2005). On the contrary, the results presented in the study are according with the studies of Centeno *et al.* (1996) in pistachio and Zhang *et al.* (2008) in pumpkin, where no relation were found between organogenesis and Z/IAA index.

ACC levels were markedly lower in peach x almond hybrids than in peach cultivars (Figure 1D) according to higher records of regenerated shoots. ACC is a precursor in the biosynthesis of ethylene. Hence, an increment of ACC content implies an increment in the ethylene production (González *et al.*, 1997). Arigita *et al.* (2003) found that the presence of ACC in the culture media reduced organogenesis when compared with the reference plants and the ethylene accumulation in the headspace was responsible for the inhibition of organogenesis and growth observed in kiwi explants.

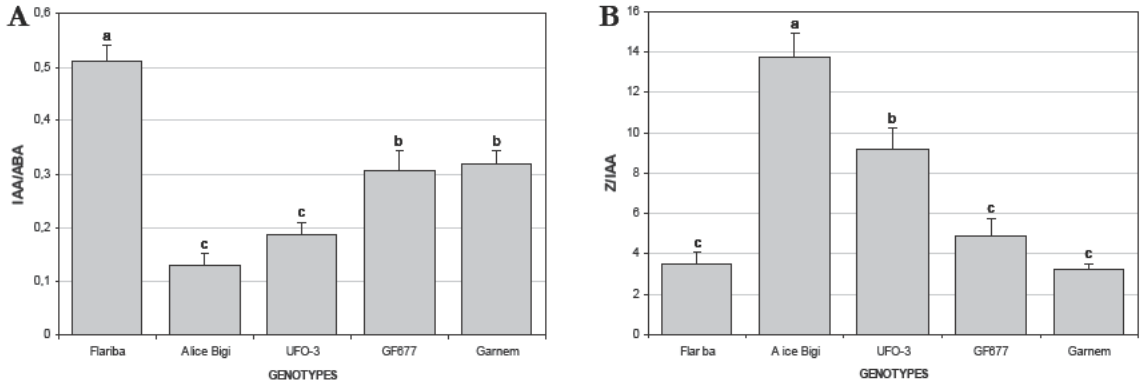

Figure 3. (a) Ratio between indole-3-acetic acid (IAA) and abscisic acid (ABA) in callus of peach (Flariba®, Alice Bigi®, UFO-3®, GF677®, Garnem®) **(b)** Ratio between zeatin (Z) and indole-3-acetic acid (IAA) in organogenic and non-organogenic callus of peach. Data represent average ± SD values. *Bars* with different lower case letters indicate a significant difference by LSD test ($P \leq 0.05$).

On the contrary, other authors have indicated an inverse relationship between organogenesis and ACC in some woody plant species (Predieri *et al.*, 1993; González *et al.*, 1997; Lakshmanan *et al.*, 1997). The SA levels (Figure 1E) found in callus of peach pointed out that medium levels of SA coincided with the genotypes, which obtained higher organogenic response, namely GF677® and Garnem®. Different response was found in the peach genotypes. SA levels in Flariba® and Alice Bigi® were the high whereas SA content in UFO-3® was the lowest amongst the genotypes. Although SA is an inhibitor of ethylene, it has been reported as a stimulator of ethylene in peach (Molasiotis *et al.*, 2005) as well as in other species (Niessen, 1994; Liang *et al.*, 1997). This controversy concerning SA leaves some unanswered questions about the precise role of SA in somatic organogenesis, which will require further studies.

Stress-related phytohormones, JA and ABA play regulatory roles in stress signalling (Ruduś *et al.*, 2009). The results obtained in the analysis of the organogenic calli showed a higher ABA content in peach cultivars than in hybrids (Figure 1F). This finding indicates a relationship between morphogenic capacity and endogenous ABA content. This is supported by the results obtained by Charrière and Hahne (1998) in experiments where an addition of exogenous ABA suppressed shoot regeneration. Under *in vitro* conditions, explants have been removed from their original tissue environment and transferred to synthetic media containing non-physiological concentrations of growth regulators and organic and in organic constituents, resulting in exposure to significant stresses

(Rai *et al*., 2011). The JA content was high in all the genotypes and no differences were found between them (Figure 1G). Plants cultured *in vitro* are exposed to high stress as they are placed on artificial media and conditions. According to the results, stress did not cause any difference in the organogenic capacity. Thus, JA does not seem to produce any direct effect related to somatic organogenesis.

In summary, we may conclude that endogenous hormonal balance in peach and peach rootstocks is crucial for successful organ development. Low levels of several hormones, namely Z, ZR, ABA, and ACC were found in the most responsive genotypes (peach rootstocks). Traditionally, cytokinins and auxins have been associated with somatic morphogenesis. However, in the present study ACC and ABA play an inhibitory role in organogenesis. This effect has been sporadically reported before, but never in *Prunus* species, these results being considered as the first studies in organogenesis and regeneration in *Prunus*. Actually, there are only a few reports in woody species about this subject. Although some hormones have been linked to organogenesis, the effects of certain of them are determined by hormone interaction or tissue sensitivity, which complicates the analysis of the results and leaves some unanswered questions. Thus, further studies are necessary to clarify the regulatory effects of the plant growth regulators added to the media and the interactions among the different hormones and their content.

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CHAPTER V: TRANSFORMACIÓN GENÉTICA

1 INTRODUCCIÓN

La transformación genética en plantas leñosas es una herramienta que permite reducir el tiempo de obtención de nuevas variedades mejoradas genéticamente, ya que la mejora clásica cuenta con unas limitaciones impuestas generalmente por la alta heterocigosidad de los cultivos, largos periodos de juvenilidad y alta auto-incompatibilidad (Pérez-Clemente *et al*., 2004). Sin embargo, las investigaciones realizadas hasta ahora no han permitido el desarrollo de un protocolo estable de transformación genética en muchas especies leñosas. El melocotón representa un ejemplo de la problemática actual en términos de transformación genética, donde los pocos trabajos publicados han sido desarrollados a partir de tejidos zigóticos (Scorza *et al*., 1990; Smigocki y Hammerschlag, 1991; Ye *et al*., 1994; Pérez-Clemente *et al*., 2004; Padilla *et al*., 2006), debido a la ausencia de un protocolo de regeneración somática desde material adulto (Gentile *et al*., 2002). La transformación desde tejidos zigóticos podría contribuir limitadamente a la mejora genética de melocotón, ya que debido a que son genotipos agronómicamente desconocidos, su utilidad se limitaría a la incorporación de genes de interés en un programa de mejora genética como fuente de germoplasma. Sin embargo, la transferencia de genes de importancia agronómica en células de melocotón derivadas de tejidos adultos, facilitaría una rápida mejora de importantes variedades comerciales (Scorza y Sherman, 1996).

Las técnicas de transformación genética desarrolladas mediante *Agrobacterium tumefaciens* son las más utilizadas debido a su sencillez y probada eficacia en muchas especies vegetales, incluyendo algunas leñosas. Así, la mayor parte de protocolos de transformación publicados en melocotón utilizan este método (Scorza *et al*., 1990; Smigocki y Hammerschlag, 1991; Pérez-Clemente *et al*., 2004; Padilla *et al*., 2006). Hasta el momento, solo hay dos trabajos de transformación genética en melocotón que han conseguido un desarrollo posterior de plantas transgénicas, aunque se comprobó posteriormente que las plantas obtenidas en uno de ellos eran en realidad quimeras (Pérez-Clemente *et al*., 2004). La elección de la cepa de *A. tumefaciens* es uno de los factores determinantes. Una de las cepas más ampliamente utilizadas para ensayos de transformación genética es la EHA 105 que porta un plásmido que confiere resistencia a antibióticos aminoglucósidos a la vez que incorpora el gen marcador de la proteína verde fluorescente (GFP), ya que este marcador presenta la ventaja frente a otros que no es necesaria la adición al medio de substratos.

Un protocolo eficiente de transformación genética requiere un alto número de células transformadas y un sistema de regeneración somática productivo (Padilla *et al.*, 2006). Los estudios desarrollados con anterioridad en esta tesis doctoral posibilitan la regeneración somática en melocotón de forma eficiente y reproducible, lo que permite avanzar en la ruta de transformación genética y centrar esfuerzos en el estudio de las variables que afectan al número de células transformadas. Con este objetivo se diseñaron una serie de ensayos para aumentar la capacidad de infección de la bacteria o la disminución de la resistencia por parte de la planta. El ajuste de distintos parámetros ha sido de gran utilidad en la elaboración y establecimiento de una vía de transformación genética en melocotón, que no ha podido concluirse con la obtención de plantas transgénicas por falta de tiempo.

2 MATERIAL Y MÉTODOS

2.1 MATERIAL VEGETAL

El material vegetal empleado para los experimentos de transformación procede de plantas multiplicadas *in vitro* en el laboratorio de cultivo de tejidos del Instituto Murciano de Investigación y Desarrollo Agrario y Alimentario (IMIDA). Estas plantas fueron establecidas a partir de segmentos nodales de árboles procedentes de la colección de variedades del IMIDA situada en la finca experimental «Torreblanca» en Cartagena, Murcia. El establecimiento *in vitro* se realizó en medio de cultivo woody plant medium (WPM) (Lloyd y McCown, 1981) y posteriormente las plántulas se propagaron en medio de multiplicación Murashige y Skoog (MS) (Murashige y Skoog, 1962) con 1 mg l^{-1} de benciladenina (BA) y 0.1 mg l^{-1} de ácido indolbutírico (IBA). Los brotes obtenidos en los macizos de proliferación se subcultivaron cada 20 días, y se mantuvieron a 25 ± 1 ºC, con luz fluorescente blanca (45 μmol m^{-2} s^{-1}, Sylvania GRO-LUX) y un fotoperiodo de 16 horas.

Los genotipos utilizados fueron Garnem®, portainjertos de melocotón resultante de la hibridación de un melocotón (*Prunus persica*, var. Nemared) y un almendro (*Prunus dulcis*, var. Garfi), el melocotón platicarpo UFO-3® y la nectarina Alice Bigi®.

Para la infección con *Agrobacterium tumefaciens* se emplearon dos tipos de material vegetal:

1) Brotes jóvenes propagados *in vitro*, de un tamaño comprendido entre 1-2 cm de longitud. Se eliminaron las hojas con la ayuda de un bisturí en cabina de flujo laminar, para facilitar la entrada de la bacteria al tallo.
2) Callos laminados inducidos en la base de los tallos, siguiendo la metodología de Pérez-Jiménez *et al.* (2012). Los callos se aislaron del resto de la planta, se laminaron con un grosor aproximado de 0.5 cm y entonces se procedió a su infección.

2.2 Estudio de la sensibilidad a kanamicina de los cultivos de callo y plantas propagadas

Las pruebas de sensibilidad a kanamicina tanto de brotes como de callos se llevaron a cabo durante 3 subcultivos. Para determinar la concentración inhibitoria que ejerce la kanamicina sobre el crecimiento del callo y la regeneración de plantas somáticas, se aplicaron concentraciones de 0, 25, 50, 75 y 100 mg l^{-1} de kanamicina al medio de cultivo y se evaluaron 50 explantos por tratamiento. Los subcultivos se realizaron cada 20 días al mismo medio de cultivo.

Tras estos 60 días se realizó una evaluación de las condiciones fisiológicas de los brotes y los callos. Esto se hizo mediante una valoración que varió de 0 a 4. Así, si se había producido la muerte del brote o el callo se le asignaba un 0 y en el caso opuesto de un brote vigoroso o un callo verde con nodulaciones (ver descripción de callo organogénico en el capítulo III) se le asignaba un 4. Además se contabilizó el número de plantas regeneradas a partir de estos explantos, en el caso de que la organogénesis hubiera tenido lugar.

2.3 Cepas bacterianas y vectores

Para llevar a cabo los experimentos de transformación se emplearon dos cepas de *Agrobacterium tumefaciens*, la EHA 105 (Hood *et al.*, 1993) y la pMP90 (Koncz and Schell, 1986). La primera es una cepa desarmada procedente de la cepa *Agrobacterium tumefaciens* A281 y la segunda de la cepa C58, que ha sido descrita como muy virulenta en *Prunus*. Las dos cepas empleadas contenían el plásmido binario pBin19-s*gfp* con el gen marcador s*gfp,* que codifica para la proteína fluorescente verde (GFP) y el gen seleccionador *nptII* que

codifica para la neomicina fosfotransferasa, enzima que confiere resistencia para los antibióticos de tipo aminoglicósidos (Figura 1).

Las cepas bacterianas procedían de un stock glicerinado a -80 ºC y se cultivaron en placa con medio Luria Bertani (LB) al que se le añadió kanamicina (50 mg l^{-1}) y gentamicina (20 mg l^{-1}) en el caso de la cepa pMP90, y kanamicina (50 mg l^{-1}) y ácido nalidíxico (25 mg l^{-1}) en el caso de la cepa EHA 105. Las placas se cultivaron en estufa 28 ºC en condiciones de oscuridad durante 48h. Estos cultivos sirvieron de punto de origen para el cultivo de noche, que se realizó en matraces de 250 ml a los que se añadieron 100 ml de medio LB con los mismos antibióticos que en el cultivo anterior correspondientes para cada una de las cepas. Los cultivos se mantuvieron en agitación a 240 rpm y 28 ºC y oscuridad durante la noche. Tras el cultivo, 45 ml de la suspensión bacteriana se centrifugaron a 3500 rpm durante 10 minutos a 15 ºC en una centrífuga 5810R (Eppendorf) y a continuación el pellet se resuspendió con 45 ml de medio MS modificado (Tabla 1). La densidad óptica de las bacterias se midió con un espectofotómetro SmartSpec Plus (Bio Rad) a una longitud de onda de 600 nm. La suspensión bacteriana se ajustó a una densidad óptica de 0,06 añadiendo el medio MS necesario.

2.4 TRANSFORMACIÓN

La infección de los brotes se efectuó en vasos de precipitados de 10 ml con 5 ml de suspensión bacteriana, en los que se introdujo la parte basal de los brotes y los callos laminados (Figura 2). Transcurrido 20 minutos, se retiraron los explantos y se eliminó el exceso de bacteria con papel secante estéril. El cocultivo se realizó en medio de cultivo MS a 25 ± 1 ºC, con luz fluorescente blanca y un fotoperiodo de 16 horas durante 72 h.

Tras el cocultivo y para eliminar la bacteria, los explantos se sumergieron en 100 ml del medio MS modificado junto con 900 mg l^{-1} de cefotaxima durante 30 minutos en agitación suave. Los brotes se transfirieron después a medio de cultivo MS con 1 mg l^{-1} de BA y 0.1 mg l^{-1} de IBA, en bote de cristal de 500 ml con 100 ml de éste medio, y los callos se cultivaron en medio MS con 2 mg l^{-1} de BA y 1 mg l^{-1} de ácido naftalenoacético (ANA) en placas Petri añadiendo en ambos casos 300 mg l^{-1} de cefotaxima y 50 mg l^{-1} de kanamicina. Los cultivos se mantuvieron a 25 ± 1 ºC y un fotoperiodo de 16 horas, efectuando subcultivos cada 20 días.

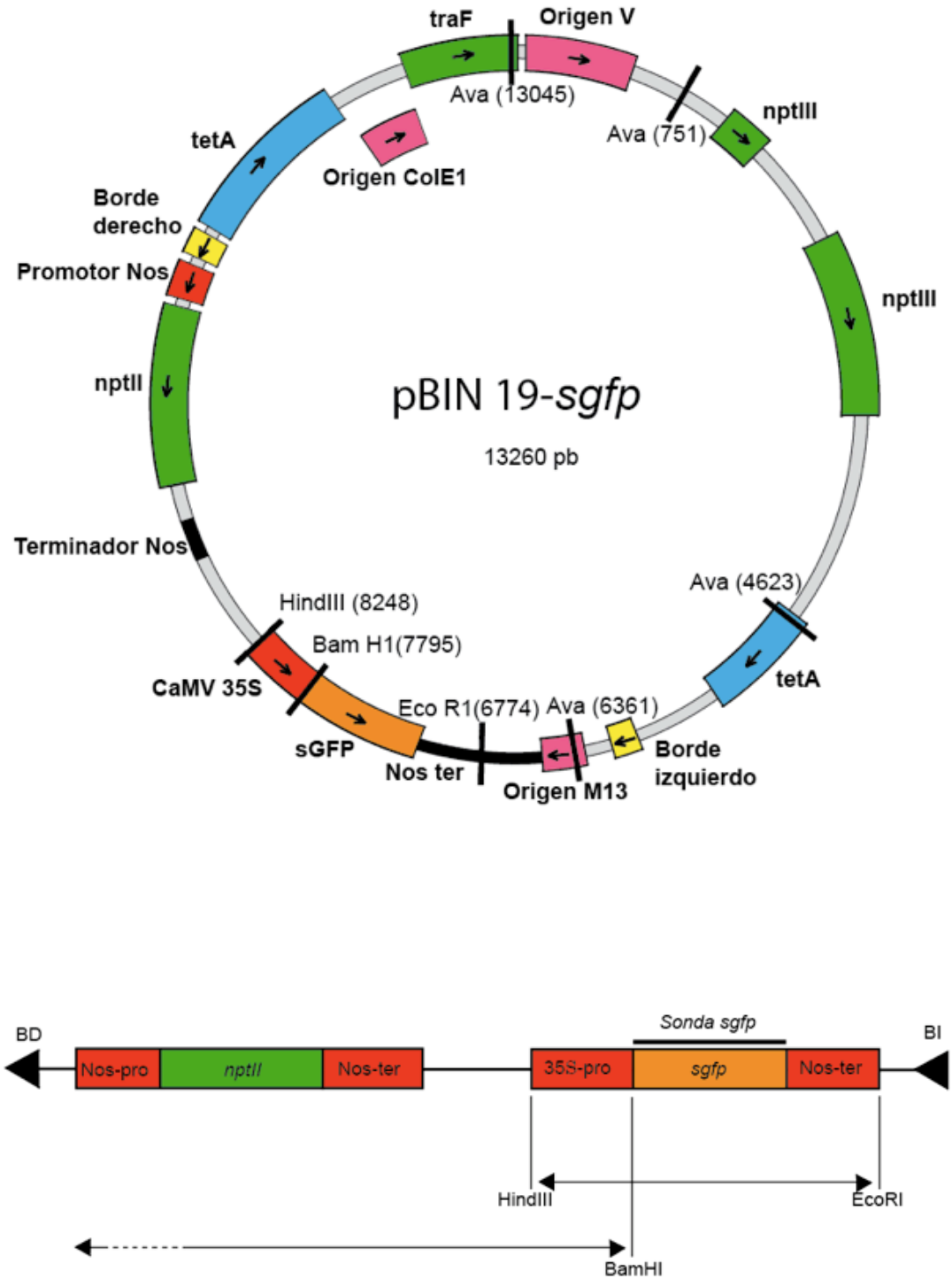

Figura 1. Plásmido pBIN19-s*gfp* y esquema del T-DNA con los puntos de restricción para las enzimas HindIII, EcoRI y BamHI. Repgresentación de la sonda s*gfp*-DIG.

Tabla 1. Medio MS modificado empleado para la resuspensión de las bacterias y para el lavado de los explantos.

Componenetes	Concentración, mg l^{-1}
Macronutrientes	
NH_4NO_3	1650
KNO_3	1900
$CaCl_2 \cdot 2H2O$	440
$MgSO_4 \cdot 7H2O$	370
KH_2PO_4	170
Micronutrientes	
KI	0,83
H_3BO_3	6,2
$MnSO_4 \cdot H_2O$	16,9
$ZnSO_4 \cdot 7H_2O$	8,6
$NaMoO_4 \cdot 2H_2O$	0,25
$CuSO_4 \cdot 5H2O$	0,025
$CoCl \cdot 6H_2O$	0,025
$FeSO_4 \cdot 7H2O$	27,8
Na_2EDTA	37,2
Vitaminas	
Myoinositol	100
Ácido Nicotínico	0,5
Piridoxina-HCl	0,5
Tiamina-HCl	0,1
Pantotenato de Calcio	0,5
Biotina	0,01
Fuente de Carbono	
Sacarosa	30000

2.5 Efecto de la adición de acetosiringona al medio de cultivo de las cepas de Agrobacterium tumefaciens

Para evaluar el efecto de la acetosiringona sobre la frecuencia de transformación, se realizó un tratamiento añadiendo 100 μM de acetosiringona al medio de cultivo de noche de bacterias y al medio de infección. Se realizaron controles con medios sin acetosiringona. Tras este tratamiento se procedió al lavado de los explantos, cocultivo con la bacteria y cultivo en medio con kanamicina.

El material vegetal empleado en este ensayo fueron brotes de las variedades UFO-3®, Alice Bigi® y Garnem®.

2.6 Efecto del tratamiento de los explantos con tween 20 previo a la infección con *Agrobacterium tumefaciens*

Para aumentar la superficie de contacto entre el explanto y la bacteria, se efectuó un tratamiento con tween 20, eliminando así la posible tensión superficial existente. El tratamiento se realizó en brotes de UFO-3® (previa infección con *Agrobacterium tumefaciens*) durante 20 minutos con una solución de 0.1% (*v*/*v*) de tween 20 en agua destilada, en agitación suave. El control se trató solo con agua destilada. Posteriormente, se realizaron 3 lavados con agua destilada estéril y se procedió a la infección con *Agrobacterium tumefaciens* anteriormente descrita.

2.7 Efecto del cultivo en oscuridad durante 60 días antes de la infección

Previa transformación, los brotes y callos se cultivaron durante 60 días en oscuridad continua en cámara climática a 25 ± 1 °C. Los controles se mantuvieron con un fotoperiodo de 16 h en luz fluorescente blanca. Tras este periodo se realizaron los ensayos de transformación según se ha descrito anteriormente.

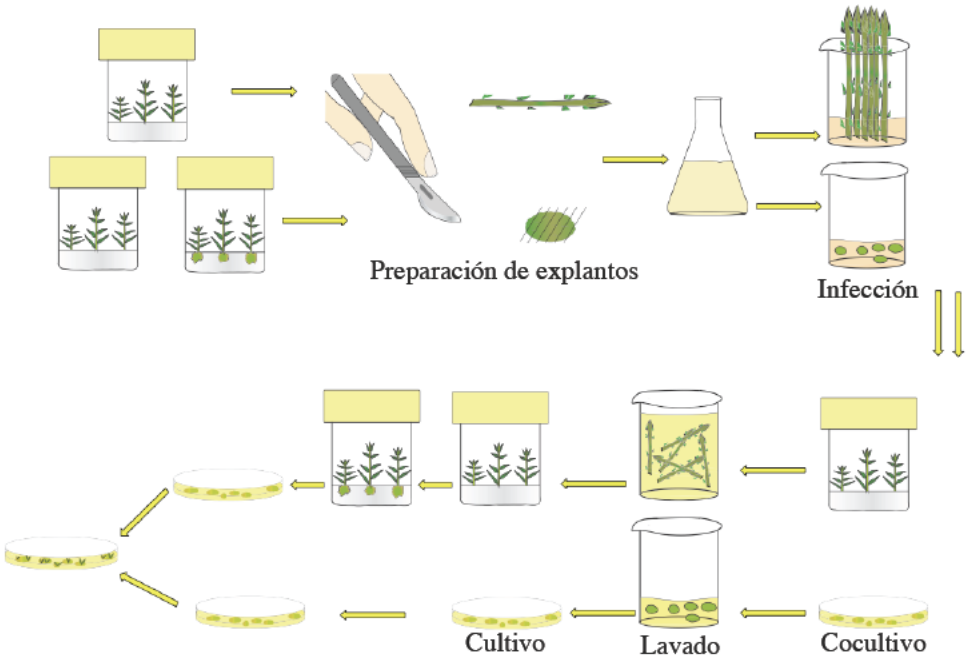

Figura 2. Proceso de transformación seguido en este capítulo para brotes y callos de las variedades UFO-3®, Alice Bigi® y Garnem®.

2.8 DISEÑO EXPERIMENTAL Y TOMA DE DATOS

Cada experimento de transformación constó de 100 explantos por genotipo (50 por cepa bacteriana) y 25 como control. Periódicamente se contabilizaron los puntos GFP con un estereomicroscópio de fluorescencia MZ 16F (LEICA). Los datos obtenidos a los 30 días se expresaron como puntos GFP totales por variedad y se analizaron mediante un ANOVA y con un nivel de significación de $P \leq 0.05$. La diferencias entre medias se realizaron mediante un test de múltiple rango de Duncan.

3 RESULTADOS Y DISCUSIÓN

3.1 ESTUDIO DE LA SENSIBILIDAD A KANAMICINA DE LOS CULTIVOS DE CALLO Y PLANTAS PROPAGADAS

La sensibilidad a kanamicina de un tejido o genotipo determinado es un factor clave en el desarrollo de cualquier nuevo sistema de transformación que proporcione resistencia a dicho antibiótico. Así, muchos autores han publicado en *Prunus* concentraciones variables de sensibilidad a kanamicina en sus experimentos con semillas inmaduras y hojas, que van desde 25 mg l^{-1} en *Prunus mume* (Gao *et al*., 2010) hasta 80 mg l^{-1} en *Prunus domestica* (Petri *et al*., 2008).

La figura 3 detalla los resultados obtenidos en términos de regeneración y estado fisiológico de los explantos expuestos a las distintos tratamientos con kanamicina. El objetivo es establecer una concentración umbral de kanamicina que, si bien no cause necrosis en el tejido vegetal, inhiba el crecimiento del callo y el desarrollo de organogénesis somática. Como puede observarse, para los tres genotipos estudiados y en los dos tipos de explanto, la concentración de 75 mg l^{-1} de kanamicina inhibió de la organogénesis, medida como número de plantas regeneradas.

Con respecto al estado fisiológico, la presencia de 50 mg l^{-1} de kanamicina en el medio de cultivo afectó negativamente al estado fisiológico tanto de brotes como de callos por lo que una exposición prolongada a esta concentración podría ser claramente perjudicial para estos explantos. A concentraciones de 75 y 100 mg l^{-1} la viabilidad de los explantos se vio seriamente comprometida (Figuras 3 y 4). A estas concentraciones de kanamicina la casi totalidad de los explantos se necrosaron.

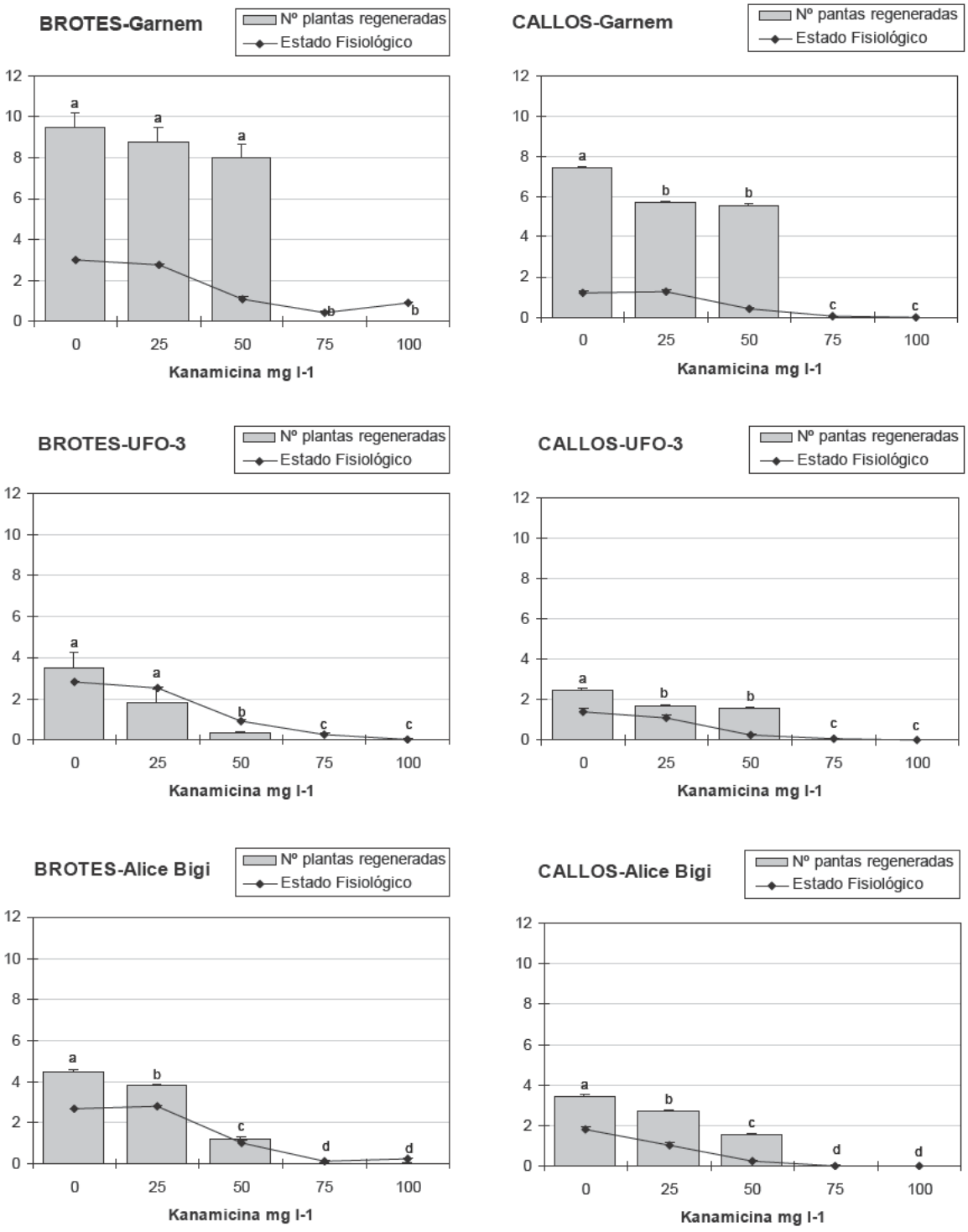

Figura 3. Regeneración y estado fisiológico de plantas y callos de Garnem®, UFO-3® y Alice Bigi® como respuesta al cultivo con distintas concentraciones de kanamicina.

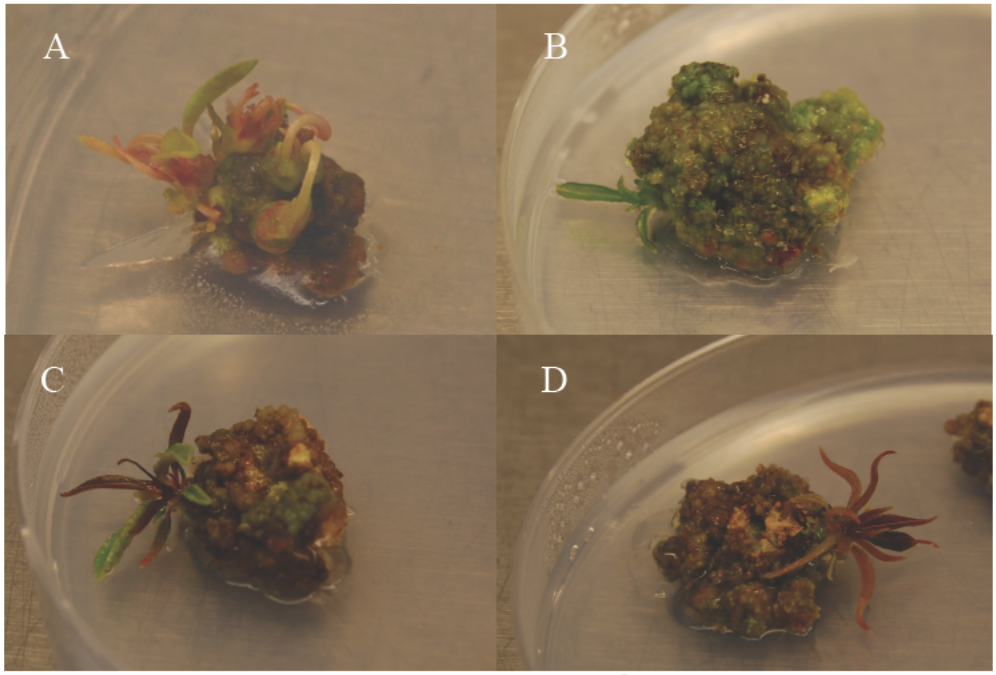

Figura 4. Efecto de la kanamicina sobre callos de Garnem®. Callos cultivados con, **(a)** 0 mg l^{-1} de kanamicina, **(b)** 25 mg l^{-1} de kanamicina, **(c)** 50 mg l^{-1} de kanamicina, **(d)** 75 mg l^{-1} de kanamicina.

En el establecimiento de una concentración de kanamicina a utilizar como agente selector de plantas transformadas, el principal problema es encontrar el equilibrio entre la selección y los efectos tóxicos de la kanamicina sobre la regeneración de tejidos (Mullins *et al.*, 1990). Así, aunque en este trabajo se ha concluido que 75 mg l^{-1} de kanamicina en medio de cultivo inhibe la regeneración de plantas somáticas, el perjuicio que produce la toxicidad de este antibiótico sobre los explantos nos hace elegir una concentración menor (50 mg l^{-1}) donde la regeneración se ve reducida sin afectar tan drásticamente a la salud de los explantos.

En los trabajos realizados por Pérez-Clemente *et al.* (2004) con tejidos embrionarios y cotiledonarios de melocotón, concentraciones mayores de 60 mg l^{-1} de kanamicina producian la inhibición de la organogénesis y la muerte del callo, y a 40 mg l^{-1} la regeneración se veía reducida. Esto coincide con los resultados obtenidos en este ensayo aunque los tejidos analizados y los genotipos fueron distintos.

3.2 EFECTO DE LA ADICIÓN DE ACETOSIRINGONA AL MEDIO DE CULTIVO DE LAS CEPAS DE *AGROBACTERIUM TUMEFACIENS*

Los ensayos del efecto de la acetosiringona en el medio de cultivo de *Agrobacterium tumefaciens* y en el medio de infección reflejaron resultados desiguales en los distintos genotipos objeto de estudio (Tabla 2) (Figura 5). Tras la infección, no se detectó ningún punto GFP en Garnem® independientemente del tratamiento o la bacteria de *Agrobacterium* que se empleara. En el caso de Alice Bigi®, la presencia de acetosiringona produjo un descenso de los positivos detectados por GFP, efecto que se produjo en ambas bacterias. Por el contrario en UFO-3®, se detectó una interacción entre cepa y tratamiento. En los explantos infectados por la bacteria EHA 105, no hubo variación estadística entre el número de puntos GFP detectado con o sin acetosiringona. Sin embargo, con la cepa pMP90 se produjo un aumento significativo del número de puntos GFP.

Tabla 2. Número de puntos GFP detectados en Garnem®, Alice Bigi® y UFO-3® infectadas en presencia de acetosiringona.

CEPA		**EHA105**		**pMP90**	
Acetosiringona, µM		0	100	0	100
Genotipo	Garnem®	0c	0c	0c	0c
	Alice Bigi®	14b	1c	20b	3c
	UFO-3®	16b	1b	19b	45a

Letras mayúsculas diferentes indican diferencias entre genotipos. Letras minúsculas diferentes indican diferencias entre tratamientos.

La acetosiringona ha sido descrita como un potenciador de la capacidad de infección de *Agrobacterium tumefaciens*, pero en algunos casos esto, dependiendo de la cepa y/o el genotipo puede ser contraproducente, ya que un aumento excesivo en la virulencia de la bacteria puede afectar seriamente a la supervivencia de genotipos más sensibles. Este hecho sería recomendable estudiarlo en más profundidad con estudios posteriores, pero lo que claramente indica este estudio es la necesidad de establecer protocolos específicos para cada genotipo. Esta podría ser la causa por la cual la acetosiringona y la elección de la cepa de *Agrobacterium* (ver tabla 3 en antecedentes) son elementos tan variables en los trabajos de transformación publicados en *Prunus*. En los trabajos de Tian *et al.* (2009), la acetosiringona

fue empleada en una concentración de 20 μM mientras que Gao *et al.* (2010) empleó una concentración de 500 μM de acetosiringona. Como puede observarse, hay una gran variación en cuanto al uso de acetosiringona como potenciador la actividad infectiva de *Agrobacterium tumefaciens*, pero ningún estudio indica la conveniencia o no de su uso en *Prunus*.

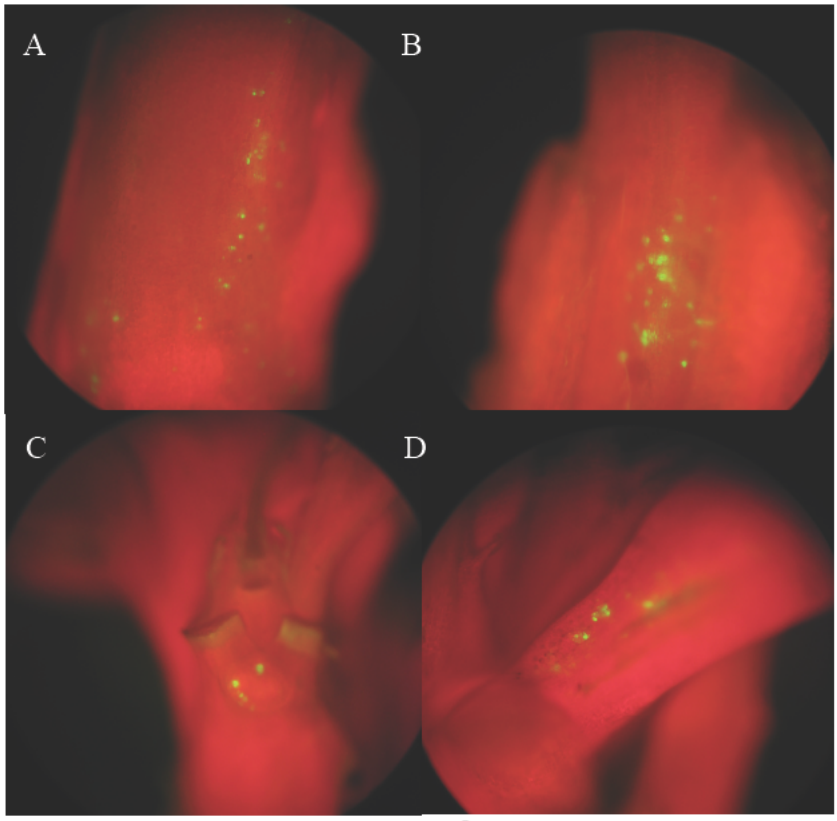

Figura 5. Presencia de puntos GFP en plantas de Alice Bigi® infectadas con la cepa de *Agrobacterium tumefaciens,* **(a)** EHA 105 y, **(b)** pMP 90, y en plantas de UFO-3® infectadas con la cepa, **(c)** EHA 105 y, **(d)** pMP 90.

3.3 EFECTO DEL TRATAMIENTO DE LOS EXPLANTOS CON TWEEN 20 PREVIO A LA INFECCIÓN CON *AGROBACTERIUM TUMEFACIENS*

La tensión superficial que se produce por la acción de la superficie cerosa de la planta podría dificultar el acceso de la bacteria a ciertas partes de la planta. Por ello, sería posible obtener un efecto positivo al efectuar un tratamiento en los explantos previo a la infección con la bacteria. Sin embargo, en contra de lo esperado, los resultados obtenidos con la incorporación de este paso previo resultaron en una disminución del número de puntos GFP (Figura 6). Kim *et al.* (2009) realizaron experimentos de transformación en *Arabidopsis* con *Agrobacterium tumefaciens* aplicando varias concentraciones de tween 20 en el

medio de infección. Estos observaron que concentraciones de tween 20 por encima del 0.05% bajaban la expresión de genes marcadores. Esto apoya los resultados obtenidos en este experimento donde una concentración de 0,1% de tween ha inhibido la infección, a pesar de las diferencias en la forma de aplicar el tratamiento. Por ello, lo más correcto sería continuar con distintos tratamientos y distintos metodologías de aplicación del tween 20, para determinar el efecto que este podría tener en la transformación con *Agrobacterium*.

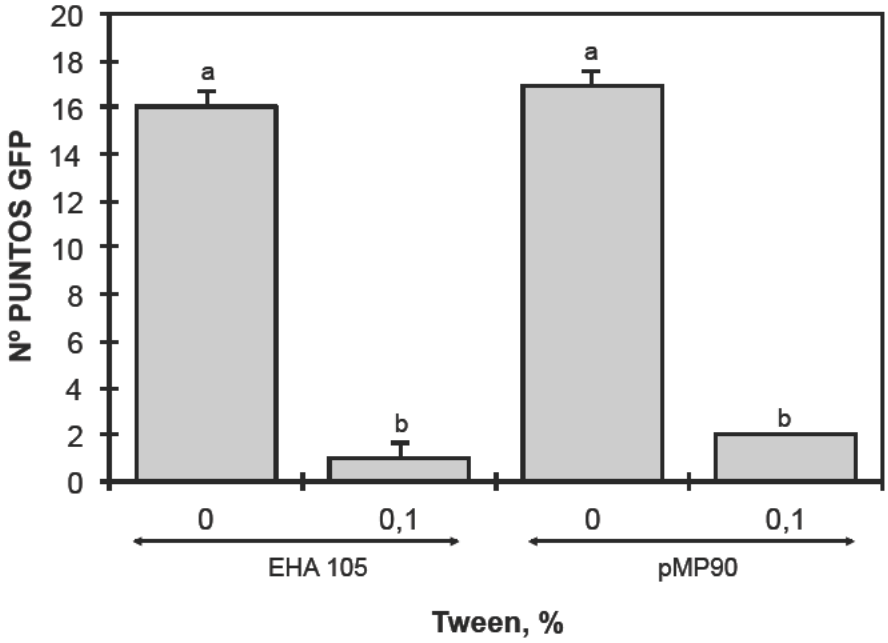

Figura 6. Efecto del tratamiento con tween 20 en brotes de la variedad UFO-3® expresados en número de puntos GFP detectados. Letras diferentes indican diferencias entre tratamientos.

3.4 EFECTO DEL CULTIVO EN OSCURIDAD DURANTE 60 DÍAS ANTES DE LA INFECCIÓN

En ensayos anteriores no se detectaron puntos GFP en los explantos infectados de Garnem®, resultados decepcionantes por ser uno de los genotipos que mayor tasa de regeneración alcanzaba (ver resultados capítulo III) . Por ello, se buscaron alternativas que pudieran posibilitar la obtención de células transformadas en este genotipo. En estudios previos (resultados no mostrados) en callos y brotes de Garnem®, se había observado que cuando estos eran cultivados en oscuridad por periodos prolongados, las células superficiales del tallo (en el caso de brotes) y del callo se etiolaban y aparentemente eran más débiles ofreciendo menor resistencia al corte con bisturí. Se pensó, que esta cambio en

las células podría favorecer la transformación con *A. tumefaciens*, por lo que se efectúo un tratamiento de cultivo en oscuridad durante 60 días previa infección.

Los resultados indican la acción beneficiosa de la oscuridad favoreciendo la transformación en este genotipo (Figura 7) (Tabla 3). Tras el proceso de oscuridad, los brotes y callos de Garnem® fueron más sensibles a la infección por *Agrobacterium*. En este caso, no se apreciaron diferencias entre cepas bacterianas en ninguno de los dos tipos de explanto empleados.

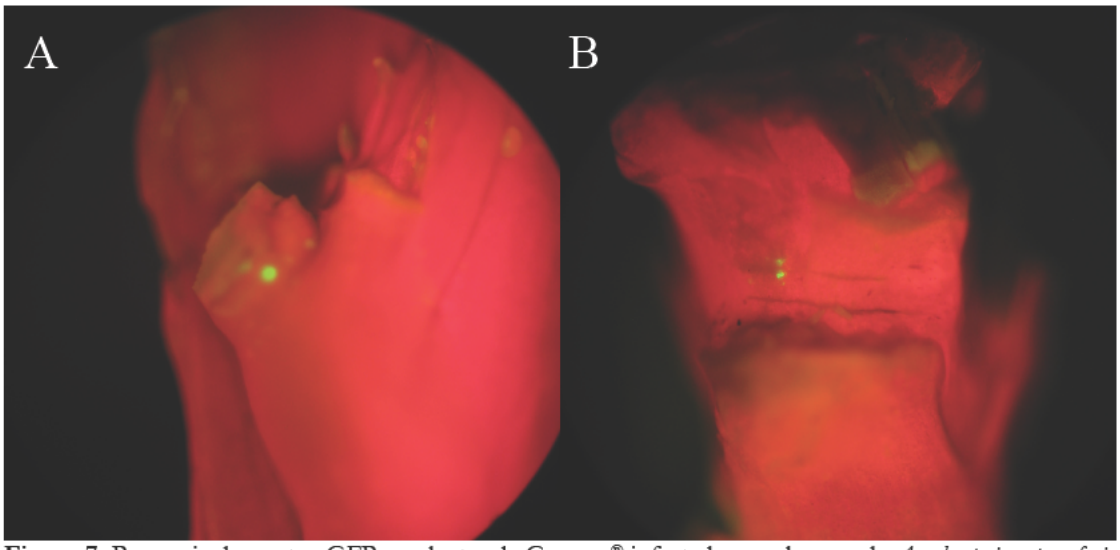

Figura 7. Presencia de puntos GFP en plantas de Garnem® infectadas con la cepa de *Agrobacterium tumefaciens*, **(a)** EHA 105 y, **(b)** pMP90.

Tabla 3. Número de puntos GFP detectados en brotes micropropagados y callos de la variedad Garnem® en condiciones de oscuridad previo ensayo de transformación.

CEPA	**EHA105**		**pMP90**	
Tratamiento	CONTROL	OSCURIDAD	CONTROL	OSCURIDAD
Brotes	0b	16a	0b	15a
Callos	0b	12a	0b	16a

Letras diferentes indican diferencias entre tratamientos dentro de una misma cepa bacteriana.

En estudios anteriormente publicados se ha estudiado el efecto de la luz durante el cocultivo (Zambre *et al.*, 2003) o durante la etapa de regeneración, pero no se han encontrado trabajos sobre un periodo de cultivo en oscuridad previo a la transformación. Dado los resultados positivos arrojados por este experimento, el paso previo por una etapa

in vitro de oscuridad podría ser una alternativa a probar en aquellos genotipos recalcitrantes en cuanto a infección por *Agrobacterium tumefaciens*.

Los ensayos presentados en este capítulo han supuesto un avance en el proceso de desarrollo de un protocolo de transformación genética de plantas de melocotón. Hasta ahora se han publicado tan solo 5 artículos de transformación de esta especie, siempre utilizando explantos derivados de semillas y obteniendo un escaso éxito en cuanto a regeneración de plantas o al desarrollo de estas. La importancia de los avances realizados y que estos se hayan efectuado en tejido adulto supone un aliciente para la continuación de esta línea de trabajo comenzada en esta tesis doctoral. Sin duda, muchos ensayos posteriores deben ser realizados para la consecución de plantas de melocotón transgénicas que mejoren las variedades que se encuentran en el mercado, pero los resultados puestos de manifiesto en este estudio pueden suponer un importante punto de partida.

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CONCLUSIONS

1. Stratification is a prerequisite in the *in vitro* culture of ovules in peach. Low temperatures have a beneficial effect in germination and also in the process of plant development, inducing an adequate growth of roots and stems.

2. Referring to carbohydrates, low concentrations of sugars benefit root and stem development during stratification. In contrast, medium concentrations of sugars induce a better elongation in roots and stems during plant growth. Glucose at the concentration of 15 g l^{-1} obtained better results in stratification and glucose during growth stage.

3. The evaluated concentrations of gibberellic acid enhanced organs development and elongation significantly when the hormone is in the culture media, but not when it was provided by soaking the seed before culture.

4. In peach, a protocol of *in vitro* embryo rescue based in two stages (stratification and culture) can enhance the quality and the amount of seedlings obtained, if each stage is provided with the adequate factors for a proper development, presented in this work.

5. Growth of *P. persica* callus is greatly influenced by the type of explant, the combination of plant growth regulators and culture media used, and the light conditions. Media composed of WPM supplemented with 2,4-D and KN induced a higher percentage of callus than the rest of the media tested. A 16 hours light photoperiod or 24 hours darkness should be applied in callus induction for different purposes. White to yellow and friable callus is obtained under dark conditions and green compact and nodular callus is produced by the effect of light. The calyx was the most productive part regarding callus induction, followed by the green explants, buds, stems and petioles. No callus was obtained from the anthers or filaments with the treatments used in this work.

6. Two cycles of 30 days on MS medium with 1 g l^{-1} of BA and 0.1 g l^{-1} of IBA, and 30 days in on MS medium with 1.5 g l^{-1} of BA and 0.1 g l^{-1} of IBA was the most efficient protocol for regenerating *Prunus persica* × *P. dulcis* hybrids and *P. persica* cultivars. It will be necessary to increase the regeneration rate if efficient peach transformation protocols are to be developed, which would ensure a high frequency of regeneration of transgenic genotypes.

7. Zeatin shows a determinant role in the induction of direct somatic embryogenesis in cotyledons of peach as well as in the Z/IAA ratio; also in ACC changes in those cotyledons that induced somatic embryos. Hence, this study confirms the importance of analysing the hormonal balance of hormone in E tissues in peach where stress-related phytohormones (ABA, JA) do not seem to produce any direct effect related to somatic embryogenesis. However, further studies are required for unequivocal explanation of the complex mechanism that leads to somatic embryogenesis in peach.

8. The endogenous hormonal balance in peach and peach rootstocks is crucial for successful organ development. Low levels of several hormones, namely Z, ZR, ABA, and ACC were found in the most responsive genotypes (peach rootstocks). As the data show, ACC and ABA play an inhibitory role in organogenesis.

9. Although some hormones have been linked to organogenesis, the effects of certain of them are determined by hormone interaction or tissue sensitivity, which complicates the analysis of the results and leaves some unanswered questions. Thus, further studies are necessary to clarify the regulatory effects of the plant growth regulators added to the media and the interactions among the different hormones and their content.

10. The genetic transformation experiments performed in UFO-3®, Alice Bigi® and Garnem® pointed out that Garnem® is more recalcitrant than UFO-3® and Alice Bigi® in terms of genetic transformation. Hence, the results concerning genetic transformation in peach depend on the genotype.

11. The effects of various concentrations of kanamycin suggest that 50 g l^{-1} is the appropriate concentration of the selection agent. Higher concentrations produced a markedly decrease in the physiological quality of the explants.

12. The acetosyringone at 100 μM included in the infection medium to test infectivity in *Agrobacterium* had different effects on the number of infected cells. This effect deserves to be studied in more detail later on.

13. Shoots treated in tween 20 added to distillate water did not show any improvement in the number of GFP points detected. In fact, the treatment was counter producer. The

number of infected cells detected was lower in the explants washed previously in tween 20 and water.

14. A period of 60 days in darkness previously to infection had a beneficial effect in transformation experiments in Garnem®. This treatment could be used in the rest of the genotypes in order to test the improvement of the transformation and its causes.

ANNEXES

ANNEX 1

Pérez-Jiménez, M., Carrillo-Navarro, A., Cos-Terrer, A. Regeneration of peach (*Prunus pérsica* L. Batsch) cultivars and *Prunus pérsica* x *Prunus dulcis* rootstocks via organogénesis. 2012. *Plant Cell Tissue and Organ Culture*. 108(1):55-6.

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ORIGINAL PAPER

Regeneration of peach (*Prunus persica* L. Batsch) cultivars and *Prunus persica* × *Prunus dulcis* rootstocks via organogenesis

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Abstract Somatic peach plants were regenerated from callus derived from the base of stem explants of the scion cultivars 'UFO 3', 'Maruja', 'Flariba' and 'Alice Bigi', and the peach × almond rootstocks 'Garnem' and 'GF677'. A protocol for organogenic plant regeneration was developed using three culture media containing different concentrations of 6 benzyladenine (BA) and indolebutyric acid to produce organogenic calli. Shoots were obtained from sliced calli after their transfer to a differentiation culture medium containing 2 mg l^{-1} BA and 1 mg l^{-1} α naphthalene acetic acid. Using this procedure, up to 29 regenerated plants per callus were obtained. The highest regeneration rate was obtained with the peach × almond rootstocks. This work provides an effective protocol that could be utilized for peach transformation research.

Keywords Morphogenesis · *Prunus* · 6 Benzyladenine · Indolebutyric acid · α Naphthalene acetic · Somatic shoots

Introduction

Plant regeneration from adult tissues is the main obstacle to obtaining transgenic peach plants. A reliable protocol is required to generate non chimeric transgenic plants (Pooler and Scorza 1995). The development of a reliable regeneration system based on mature tissues is a prerequisite for application of transformation techniques for improvement of woody species (Litz and Gray 1992; Liu and Pijut 2008, 2010), and might be particularly useful to improve biotic and abiotic stress resistance and fruit quality (Srinivasan et al. 2004). Plant regeneration is affected by many factors, such as genotype, culture medium, plant growth regulators, agar, type of explant and light conditions. For example, cytokinins are major factors in the induction of somatic organs (George 1993; Magyar Tabori et al. 2010). The physiological and chronological age of explants and the in vitro culture period can influence organ formation (Hammerschlag et al. 1985). In addition, the difficulty of regenerating plants from mature tissues of woody plants is well established (Smigocki et al. 1991).

Peach is one of the most recalcitrant species with regard to in vitro regeneration (Bhansali et al. 1990; Padilla et al. 2006). Successful regeneration of peach plants is rare despite the use of juvenile explants as starting material. Immature seeds have been used most frequently as a vegetative explant in peaches (Meng and Zhou 1981; Hammerschlag et al. 1985; Scorza et al. 1990; Bhansali et al. 1991; Smigocki et al. 1991; Svircev et al. 1993; Pérez Clemente et al. 2004). Adventitious shoots have been regenerated successfully in peach from leaf explants excised from in vitro shoot apex cultures (Gentile et al. 2002). Most authors (e.g., Declerck and Korban 1996) have used segments of vegetative organs, such as leaves previously excised from the plant, for callus induction. Mezzetti et al. (2002) reported a similar protocol for grape (*Vitis vinifera*) to that presented in this study, whereby callus is induced prior to excision of the explant from the plant.

This paper describes an effective protocol for in vitro regeneration of peach via organogenesis. Three treatments comprising different concentrations of cytokinin and auxin in the culture medium were compared. A histological examination of the organogenic calli was carried out in order

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to monitor the regeneration process and to confirm the neo formation of shoots.

Materials and methods

Plant material

Plant material was obtained from 4 year old peach trees grown at the Torreblanca experimental field station of the Instituto Murciano de Investigación y Desarrollo Agrario y Alimentario (IMIDA), Murcia, Spain. Nodal segments of the scion cultivars 'UFO 3', 'Maruja', 'Flariba' and 'Alice Bigi', and of the peach × almond rootstocks 'Garnem' and 'GF677', were collected and transferred to the tissue cul ture laboratory.

In vitro establishment

The nodal segments were sterilized in a solution of 2% (v/v) sodium hypochlorite and 0.1% (v/v) Tween 20 for 2 h. Shoot cultures were established in vitro and subcul tured monthly on M1 medium (Table 1) for 3 months. M1 medium was composed of Murashige and Skoog (MS) salts (Murashige and Skoog 1962), 6% (w/v) sucrose and 0.7% (w/v) of Plant Propagation Agar (Pronadisa®). The pH was adjusted to 5.7 with 0.1 N KOH prior to autoclaving at 122°C (1.1 kg cm^{-2}) for 16 min. The proliferating shoots were cultured in climatic chambers at 25 ± 1°C and with a 16 h light period (45 µmol m^{-2} s^{-1}; Sylvania Gro lux fluorescent tubes).

Callus induction and regeneration

The organogenic calli were obtained from the base of proliferation clusters induced on M1 medium (Table 1). Prior to culturing on organogenic medium (OM), the calli were isolated and sliced. Sections were approximately 3 mm thick and were divided equally between longitudinal and transversal cuts to rule out position effects on regen eration capacity. The sections were exposed to three treatments (Fig. 1) consisting of different 6 benzyladenine (BA) and indolebutyric acid (IBA) concentrations and subculture cycles. Treatment 1 (T1) consisted of 30 days on M1; treatment 2 (T2) consisted of two cycles, 30 days on M1 and 30 days in on M2; and treatment 3 (T3) con sisted of 30 days in on M1, 30 days in on M2 and 30 days in on M3. After treatment, the regeneration capacity was tested on OM medium for all treatments (Table 1; Fig. 1).

Table 1 Composition of the culture media used during the induction (M1, M2, M3), regeneration (OM), elongation (EM) and rooting (RM) phases

	M1	M2	M3	OM	EM	RM
Salts	MS	MS	MS	MS	MS	MS
NAA (mg l^{-1})	–	–	–	1	–	–
IBA (mg l^{-1})	0.1	0.1	0.1	–	–	1.5
BA (mg l^{-1})	1	1.5	2	2	–	–
Sucrose (g l^{-1})	30	30	30	30	30	30
Agar (g l^{-1})	7	7	7	7	7	7

MS Murashige and Skoog salts, *NAA* α-naphthalene acetic acid; *IBA* indolebutyric acid; *BA* 6-benzyladenine

Elongation and rooting

Regenerated plants were separated and transferred to elongation medium (EM) to increase their survival capacity prior to acclimatization (Table 1). Explants approximately 5 cm long were transferred to rooting medium (RM) (Table 1), and acclimated when the roots were at least 2 ± 0.2 cm long.

Experimental design and data collection

Data were recorded for 25 calli from each cytokinin con centration (total 75 calli). Treatments comprised cut ori entation (longitudinal vs transversal) and cultivar. Data on shoot production (number of shoots per cluster) was recorded prior to sectioning. Other measured parameters were frequency of organogenic calli (FOC), organogenesis rate (OR) and percentage of callus with somatic shoots. Each experiment was repeated three times. All data were subjected to ANOVA and the significance ($P \leq 0.05$) of differences between mean values was tested using Dun can's new multiple range test.

Histology

Slides of the regenerating calli were prepared for histo logical examination. After culture for 3 months on OM medium, 15 calli were sampled to observe their tissue organization. The fixation procedure was based on that of Jensen (1962). The calli were stained with hematoxylin and eosin. Explants were dipped in FAA fixative (4% (v/v) formaldehyde, 70% (v/v) acetic acid and 70% (v/v) alco hol; 1:1:18) for 24 h. The fixed material was dehydrated in a tertiary butyl alcohol (TBA Merck®) series from 50 to 100%. The samples were transferred four times to liquid paraffin at 60°C. After 24 h, the samples were embedded in paraffin blocks, and 10 µm thick sections were cut with a

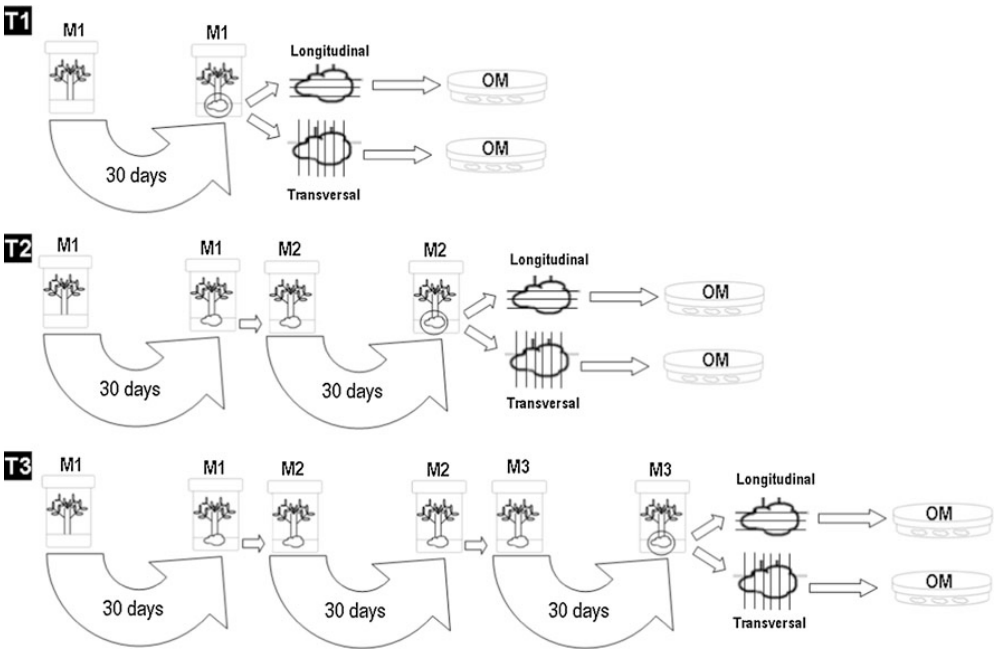

Fig. 1 Treatments for callus induction and shoot regeneration. M1 = MS + 0.1 mg l ^{1} IBA + 1 mg l ^{1} BA. M2 = MS + 0.1 mg l ^{1} IBA + 1.5 mg l ^{1} BA. M3 = MS + 0.1 mg l ^{1} IBA + 2 mg l ^{1} BA. OM = MS + 1 mg l ^{1} ANA + 2 mg l ^{1} BA

LEICA RM 2155 microtome and mounted on SuperFrost slides using TESPA glue. Hydration was carried out with xylol and decreasing concentrations of alcohol. The sec tions were stained with hematoxylin and eosin and dehy drated in xylol and increasing concentrations of alcohol. Samples were again hydrated using xylol and increasing concentrations of alcohol. Coverslips were mounted in DPX (Leica Microsystems), and the slides were observed under a Olympus SZX10 stereomicroscope and Olympus BH2 RFCA microscope at 4×, 10× and 40× magnifica tion. Motic Images Advanced 3.2 software was used to capture digital images.

Results

Callus induction

Calli were induced from somatic cells during the first week after transfer to the induction media. The calli appeared at the stem base where the stem was in contact with the induction medium (Fig. 2a). During the next 2 weeks, the calli grew in size, and became green and more compact. During the fourth week of culture, green compact nodules appeared on the surface of the calli.

Adventitious shoot organogenesis

After transfer of callus slices to OM, the calli turned darker green and nodule growth was stimulated. Initially, two zones in the slices could be differentiated: an internal white zone and an external zone covered by green nodules (Fig. 2b, c). After culture on OM for 1 week, shoots started to develop from the nodules on the callus surface (Fig. 2d i), but some of the nodules gave rise to shoots after only the third or fourth week. Organogenesis began 10 days after transfer to OM and continued for up to 3 months.

Cultivar response

Differences between the cultivars were statistically signif icant ($P < 0.05$) and two groups were distinguishable, namely peach × almond hybrids and peach cultivars. The highest regeneration rates were obtained for the hybrids (Table 2). Most 'GF677' and 'Garnem' calli were orga nogenic and more than 80% of the calli produced shoots; 29 and 10 shoots per callus, respectively, were the maxi mum number of new shoots obtained for the hybrids. In contrast, the peach cultivars produced no more than one or two shoots per callus in all of the treatments, and showed lower FOC values than the hybrids (Table 2). Differences between cultivars were also evident in the proliferation rate

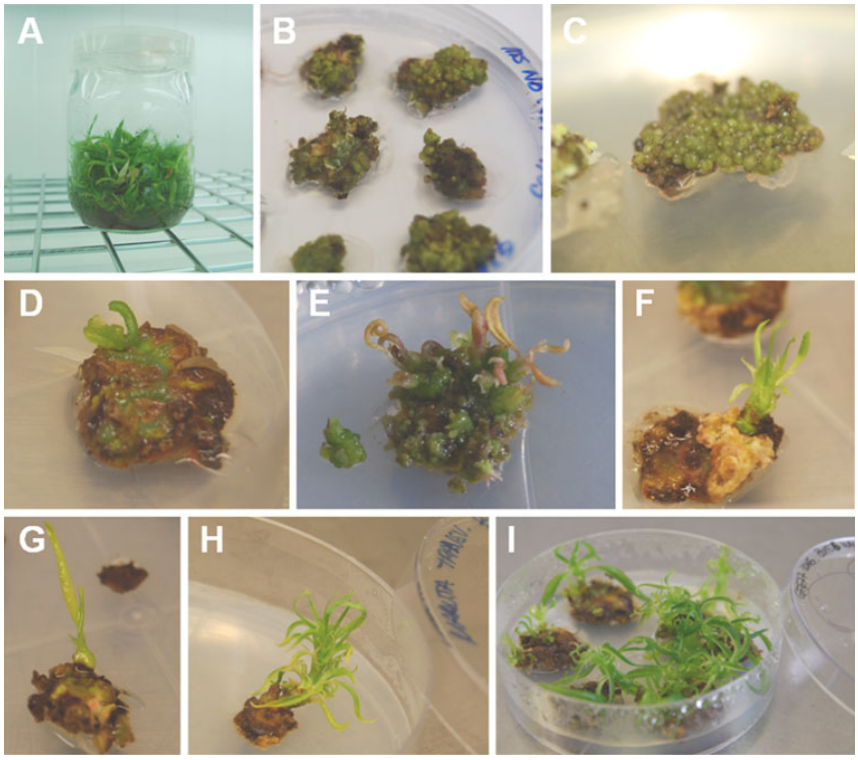

Fig. 2 Adventitious shoot regeneration from in vitro nodal segment explants of peach cultivars and peach × almond hybrids. **a** Proliferation cluster of 'GF677' in vitro. **b** Slices of 'Garnem' callus in OM. **c** Callus formation on a slice of 'Garnem' in OM. **d** Adventitious shoot on 'Alice Bigi' callus. **e** Adventitious shoots on 'Garnem' callus. **f** Adventitious shoot on 'UFO-3' callus. **g** Adventitious shoot on 'Flariba' callus. **h** Adventitious shoot on 'Maruja' callus. **i** Adventitious shoots on 'GF677' calli

Table 2 Frequency of organogenic callus (FOC) formation and maximum number of shoots produced per callus in peach cultivars (pc) and peach × almond hybrids (h)

Values in each column followed by the same letter are not significantly different ($P < 0.05$)

Genotype	FOC (%)				Max. shoots number per callus
	T1	T2	T3	Mean	
GARNEM (h)	84.0	91.6	66.6	80.8a	10a
GF677 (h)	80.0	91.6	75.0	82.2a	29a
FLARIBA (pc)	0	8.3	0	2.8b	2b
MARUJA(pc)	0	16.6	0	5.6b	1b
UFO-3 (pc)	0	25.0	0	8.3b	2b
ALICE BIGI (pc)	0	8.3	8.3	5.6b	1b
Mean	27.3b	40.2a	24.9b		

(Fig. 3). The highest frequency of organogenic calli was obtained from the base of the most proliferative callus clusters, as demonstrated by the low positive Pearson's correlation coefficient (r 0.463).

Treatment response

The proliferation rate increased progressively from T1 to T3. The T3 treatment also showed the highest regeneration rate (Fig. 4a). The differences between the treatments were significant ($P < 0.05$). Regeneration was observed in calli from the clusters that showed the highest proliferation rates (Fig. 4b). The T1 treatment induced organogenesis in the hybrids but did not induce shoot regeneration in any of the peach cultivars (Fig. 3). T2 induced organogenesis in all of the genotypes and yielded the highest organogenesis rate. Treatments T2 and T3 induced identical numbers of shoots in 'Alice Bigi'

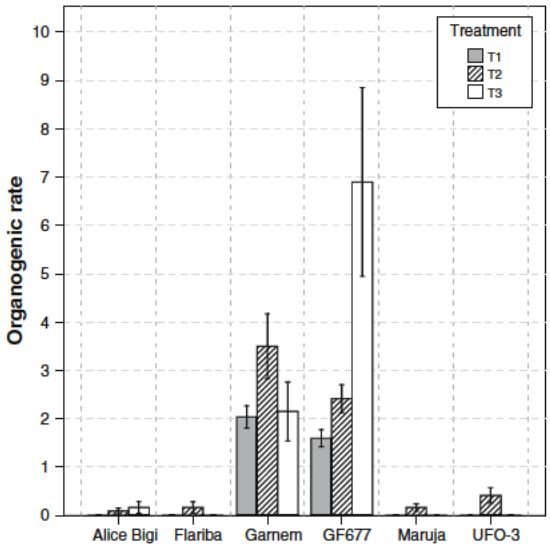

Fig. 3 Genotype and treatment effects on organogenesis rates in peach cultivars and peach × almond hybrids. *Vertical bars* represent the standard deviation

(Table 2). T3 showed the lowest mean regeneration rate for five of the six genotypes.

Effects of cut orientation

The mean regeneration rates obtained for longitudinal and transversal explant sections did not differ significantly in any of the treatments or cultivars (Table 3; Fig. 5).

Histological observations

The picture sequence in Fig. 6 was taken under the stereo microscope or microscope. Note the different structures involved in somatic plant development. During callus for mation, undifferentiated cells were produced from the stem base. Hence, no organized tissue is discernible in Fig. 6a. The callus formed did not preserve any feature of the stem cells, in case a bud was present at the base of the stem. In Fig. 6a, the callus (ca) produced two shoots (sh), two mer istems and several leaf primordia. The meristemoid (Fig. 6b) comprised a group of meristematics cells that are smaller than the typical callus cells and contain a large central nucleus, which increased the nucleus: cytoplasm ratio of the cell. The external cells form the tunica and the remaining cells give rise to the corpus. The differentiating shoot (Fig. 6c) comprised two external cell layers, the tunica, and an internal mass of cells, the corpus. Several leaf primordia can be seen growing from the surface of the callus in Fig. 6d.

Discussion

This study describes an effective protocol for the in vitro generation of somatic peach plants via organogenesis, The procedure is essential for the genetic transformation of the species and enables one of the major barriers hindering peach genetic improvement programmes to be overcome. Although peach transformation techniques have been per fected, an effective regeneration protocol was still lacking.

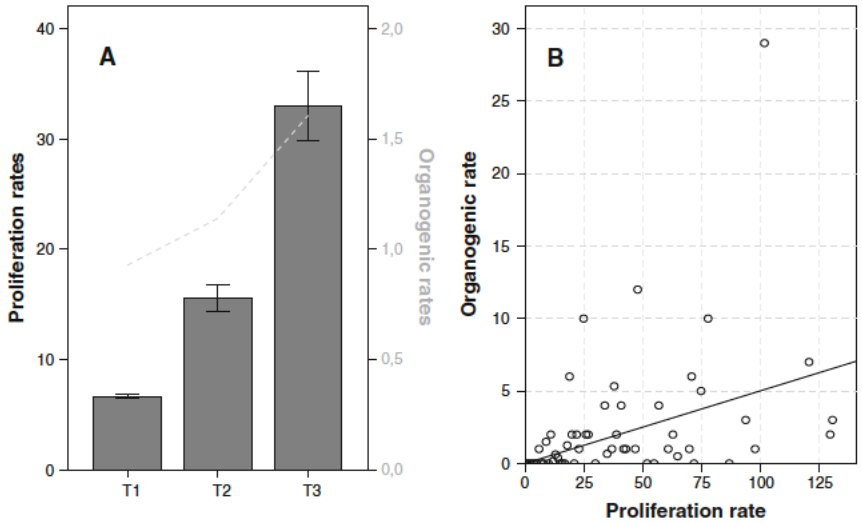

Fig. 4 Relationship between number of shoots obtained per cluster (proliferation rate) and the percentage of callus with somatic shoots (organogenesis rate). **a** Histogram of organogenesis rates in each treatment. The *dotted line* represents the proliferation rate. **b** Regression of organogenesis rate and proliferation rate

Table 3 Effect of culture medium and explant cut orientation on frequency of organogenic callus (FOC) formation in peach cultivars and peach × almond hybrids

Genotype	Treatment	FOC (%)	
		Longitudinal	Transversal
GARNEM	T1	75.2a	92.1a
	T2	100a	80.5a
	T3	50.3b	83.4a
	Mean	75.2a	85.3a
GF677	T1	75.2a	85.5a
	T2	100a	83.1a
	T3	67.6a	83.1a
	Mean	80.9a	83.9a
FLARIBA	T1	0a	0a
	T2	17.1a	0a
	T3	17.1a	0a
	Mean	11.4a	0a
MARUJA	T1	0a	0a
	T2	17.2a	17.1a
	T3	17.2a	17.1a
	Mean	11.5a	17.1a
UFO-3	T1	0a	0a
	T2	0b	50.2a
	T3	0b	50.4a
	Mean	0b	50.2a
ALICE BIGI	T1	0a	0a
	T2	0a	17.2a
	T3	0a	17.2a
	Mean	0a	17.2a

Values in the same column followed by the same letter are not significantly different ($P < 0.05$)

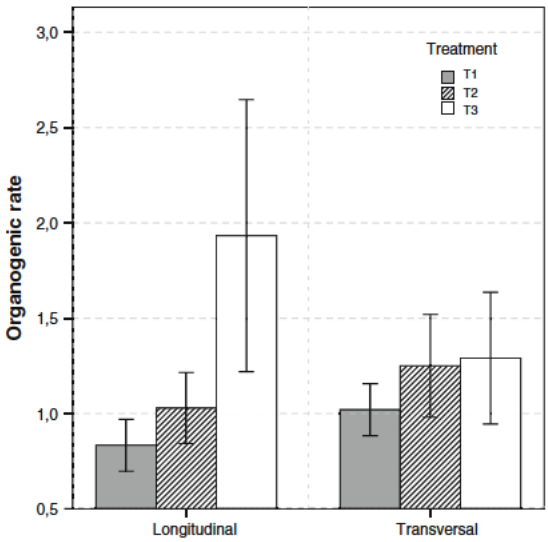

Fig. 5 Effect of cut orientation on regeneration rates in the different treatments. *Vertical bars* represent the standard deviation

Previous studies of in vitro regeneration of peach carried out by other research groups used material derived from seeds, which is of scientific but not agronomic utility. To our knowledge, the only previous study that successfully regenerated peach plants using adult tissues is that of Gentile et al. (2002), but reproduction of these authors' results has proved difficult.

The protocol described here to obtain shoots by organogenesis utilized calli that developed at the base of the proliferation cluster. New calli developed around the cut surfaces of the sections, as previously reported (Declerck and Korban 1996). The callus induction process was initiated at the base of the stem in the cut surface where the cells were in contact with the culture medium and plant growth regulators. Callus started growing, producing a large clump of callus at the base of the explant. Green, compact callus with nodules on its surface formed, as described by Gentile et al. (2002). The organogenic callus developed as a result of the gradually increasing cytokinin content (Mezzetti et al. 2002) and increasing endogenous hormone concentration produced by the plant while it was growing. Endogenous and exogenous regulators induce somatic shoot formation.

In this type of organogenesis system, new shoots are formed following suitable hormonal treatment and differentiate in three organogenesis stages: competence (Howell et al. 2003), determination (Gahan and George 2008) and morphogenesis (Sugiyama 1999). This type of protocol, involving proliferation clusters, has been successfully applied to other plant species such as *Vitis vinifera* (Mezzetti et al. 2002). Of the different cytokinin treatments applied, T2 yielded the highest organogenic callus frequency and number of shoots per explant. This treatment consisted of two consecutive culture cycles with an increasing concentration of BA, which has been used as an organogenic regulator in several species, for example, in sour and sweet cherry (Tang et al. 2002), pistachio (Tilkat et al. 2009), *Prunus persica* × *P. davidiana* (Zhou et al. 2010), *Colocynthis citrullus* (Ntui et al. 2009), and blackberry (Gupta and Mahalaxmi 2008). The T2 treatment was successful for all of the peach cultivars and *P. persica* × *P. dulcis* hybrids studied, and provided a mean of 40.2% organogenic calli. According to Svircev et al. (1993), peach regeneration rates depend on genotype. The fact that the two hybrids included in the present study showed a similarly high regeneration response to hormones as the peach cultivars might reflect the parentage of the hybrid genotypes. Peach × almond hybrids demonstrate hybrid vigor in general and both GF677 and Garnem have been described as particularly vigorous

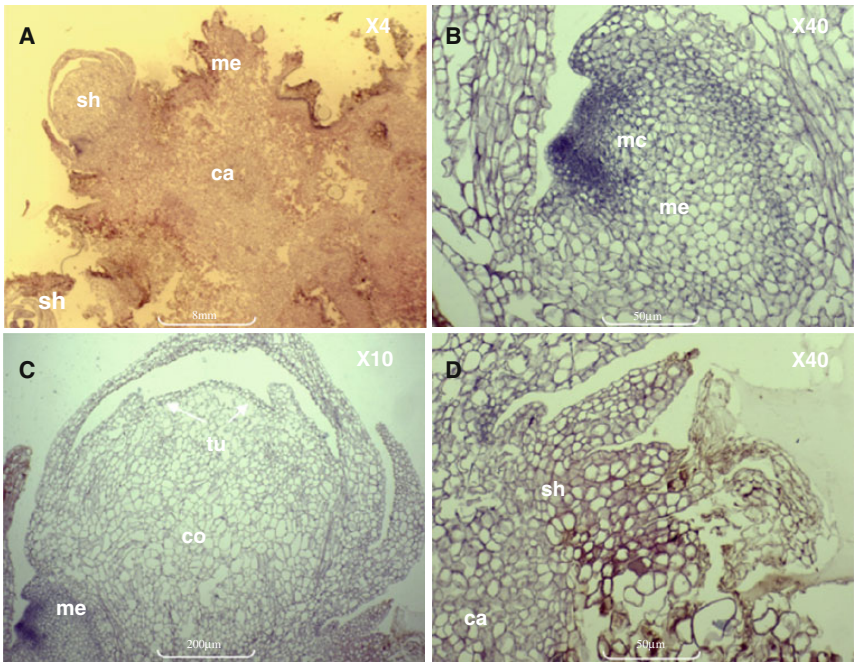

Fig. 6 Histological sections of organogenic callus and adventitious shoots of the peach × almond hybrid rootstock 'Garnem'. **a** Transverse section of the callus, presenting two shoots. **b** View of the developing meristemoid. **c** Longitudinal section of the emerging bud. **d** Leaves and a new shoot emerging from the callus. ca callus, *co* corpus, *mc* meristematic cells, *me* meristemoid, *sh* shoot, *tu* tunica

genotypes (Felipe 2009). Although almond is one of the most recalcitrant species for in vitro culture (Ainsley et al. 1999), the two peach × almond hybrids showed the highest regeneration rates in the present study. Peach is highly inbred, and inbreeding depression (Charlesworth and Charlesworth 1987) might be partially responsible for the low regeneration rates observed.

The differences in organogenesis between the four peach cultivars were not significant and the fact that they were typologically distinct (representing peach, pavia, nectarine and flat peach types) did not affect their regen erative capability. However, this finding must be corrobo rated using additional cultivars. Similarly, the type of cut (longitudinal vs. transversal) did not affect the organogenic capability of the cultivars. Although transversal sections (except in T3) were slightly more effective than longitu dinal sections, the difference was not statistically significant.

An important parameter was the relationship between the organogenesis rate and the proliferation rate of the clusters from which the explants arise, which indicates that it would be convenient to preselect the most proliferative clusters to obtain calli with higher proliferation rates. The endogenous levels of the hormones supplied by the plant appear to facilitate peach organogenesis. In previous studies the hormone levels have not been suitably adjusted, and it is advisable to further investigate endogenous hor mones in vitro to identify which plant growth regulators the plant is supplying to the organogenic callus during callus formation by the explant.

With the above in mind, we wondered whether the regenerated calli might be preformed in the callus obtained. To clarify this possibility, histological examination of longitudinal and transversal sections was undertaken. In all cases, shoot neoformation took place in the external part of the callus, where no vascular connections with the stem are present, demonstrating that axillary buds are not embedded in the callus. Moreover, given the large number of shoots that differentiated from some calli (up to 29 per callus) (Table 2), it is impossible for the shoots to be preformed at the base of the stem. The histological examinations were helpful to closely observe organ development. According to Amorós et al. (1991) cellular differentiation results in the typical structure of the differentiating shoot, which comprises the tunica and corpus. Hematoxylin deeply stains nuclei and cytoplasm; therefore meristematic cells were more intensely stained. These cells are involved in a strong process of multiplication, presenting a large nucleus and small size. As Ghimire et al. (2010) described, shoots differentiate from cells on or near the callus surface, and those cells in closest contact with the culture media.

The T2 treatment was the most efficient protocol for regenerating *Prunus persica* × *P. dulcis* hybrids and *P. persica* cultivars in the present study. It will be neces sary to increase the regeneration rate if efficient peach transformation protocols are to be developed, which would

ensure a high frequency of regeneration of transgenic genotypes. The protocol described herein should allow the development of improved transformation protocols for peach and other *Prunus* species.

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ANNEX 2

Pérez-Jiménez, M., López-Soto, M.B., Cos-Terrer, J. In vitro callus induction from adult tissues of peach (*Prunus persica* L. Batsch). *In Vitro Cellular and Developmental Biology Plant* . Accepted 5 September 2012**.** DOI: 10.1007/s11627-012-9466-8.

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PLANT TISSUE CULTURE

In vitro callus induction from adult tissues of peach (*Prunus persica* L. Batsch)

Margarita Pérez Jiménez · M. Belén López Soto · José Cos Terrer

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Abstract We describe an efficient protocol for callus induction from adult tissues of *Prunus persica* (L.) Batsch. Three different commercial peach genotypes, Early May®, Zise May®, and UFO 3®, plus three other genotypes from hybrid crosses performed in February 2006, PS108, PS208, and PS708, were used in the study. Thirteen explant treatments were tested using nine different plant parts. Murashige and Skoog and woody plant medium salts were assayed with several concentrations of 2,4 dichlorophenoxyacetic acid (2,4 D), kinetin (KN), and thidiazuron, and two different photoperiods were tested, a 16 h photoperiod or continuous darkness. In terms of the quantitative response, two parameters were assessed: the number of days to callus induction and relative callus growth recorded after 30 d. Woody plant medium supplemented with 2,4 D and KN significantly increased the rates of callus induction in the majority of treatments. And no significant differences among the *P. persica* genotypes were found. The explants derived from stem and calyx produced up to 85 and 96 % callus induction, respectively. The protocol described here could be used for efficient callus induction in a range of *Prunus* spp.

Keywords *Prunus* · Nectarine · WPM · 2,4 D · TDZ · KN

Introduction

Callus is an amorphous and dedifferentiated tissue composed of disorganized cells. It may be produced naturally in response to insect or microorganism attack or stress (George 1993). Several *in vitro* biotechnological techniques have been developed, all of which require a reliable protocol to produce a responsive cell mass. Unorganized cells of *Prunus* spp. have traditionally been cultured for protoplast fusion of different individuals, somatic hybridization (Hidano and Niizeki 1988), to obtain haploids (Peixe *et al.* 2004), or for induction of tolerance to low temperatures (Arora and Wisniewski 1995). Callus induction has also been used for genetic transformation (Scorza *et al.* 1994) and adventitious regeneration (Gentile *et al.* 2002), which is the initial phase in a transformation protocol.

Peach is one of the most widely consumed fruits in the world, but its recalcitrance in many biotechnological processes has hindered the advance of *in vitro* techniques. For most purposes, *in vitro* callus establishment is important as an intermediate step in peach biotechnology. Most of the advances made in peach have used embryo derived explants. The main disadvantage of developing a protocol from seed derived material is that each genotype is unique and not a clone of the parent (Abbott *et al.* 2008). Development of these biotechnological tools from mature tissues is important for the improvement of desirable commercial cultivars that have been selected for beneficial features, which may not be present in naturally produced seeds. Only a few authors have developed somatic regeneration protocols using adult peach material (Gentile *et al.* 2002; Pérez Jiménez *et al.* 2012).

Many factors affect callus induction as well as its growth and development *in vitro*, namely, the type of explant tissue, quality and type of light and photoperiod conditions, plant growth regulators, culture media, gelling agent, pH, temperature, and many others. Plant growth regulators particularly influence callus induction; a phase in which auxins play a major role by inducing callus proliferation and development (Paris *et al.* 2004). Different types of callus are frequently developed for different purposes. A white and globular callus

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has been used for embryogenesis (López Pérez *et al.* 2005; Hammerschlag *et al.* 1985), while green and nodular calli are used for organogenesis (Gentile *et al.* 2002; Pérez Jiménez *et al.* 2011). Thus, a thorough study of all the factors involved is important for determining the choice of protocol for the type of callus required.

This work aimed to develop an efficient and reproducible protocol for obtaining callus from adult material in peach, by comparing different types of explant, culture media, growth regulators, and culture conditions. The results will be useful to develop further studies, to regenerate plants, protoplast cultures, and to obtain metabolites from *in vitro* cultures.

Materials and Methods

Plant material. Plant tissues were collected from 4 yr old peach (*Prunus persica* L. Batsch) trees grown at the Torreblanca experimental field station of the Instituto Murciano de Investigación y Desarrollo Agrario y Alimentario, Murcia, Spain. Three commercial varieties Early May® (nectarine), UFO 3® (flat peach), and Zise May® (peach) and another three preselected varieties from a peach breeding program using hybrid crosses PS108 (Early May® × N 292), PS208 (Early May® × UFO 3®), and PS708 (Zise May® × Early May®) were used as explant sources. The plants were watered daily using a drip irrigation system with 700 $m^3 ha^{-1}/mo$ at the time of sampling. The samples for callus induction experiments were taken from young branches producing their first shoots after flowering in April 2010.

In vitro culture establishment. Branches and flowers in the balloon stage from the selected genotypes were disinfected in a solution of 2 % (*v/v*) sodium hypochlorite and 0.1 % (*v/v*) Tween 20 for 2 h. After disinfection, the plant material was transferred to a laminar flow cabinet to separate the plant organs. Six sections of vegetative organs (fully expanded leaf blade, petiole, juvenile leaf, stem, terminal buds, and axillary buds) and three reproductive organs (calyx, petals, and stamens) were selected and cultured as follows: leaf explant with the abaxial leaf surface facing the medium, with and without midvein; leaf explant with the adaxial leaf surface facing the medium, with and without midvein; approximately 1 cm long stem explants plated in an upright or inverted position; juvenile leaf; terminal bud; axillary bud; petiole; calyx; petals; and stamens (Fig. 1).

Culture media. The explants were cultivated on four different media: Murashige and Skoog medium (MS; Murashige and Skoog 1962) supplemented with 1.2 $mg l^{-1}$ of 2,4 dichlorophenoxyacetic acid (2,4 D) and 1 $mg l^{-1}$ of kinetin (KN, MS DK); MS supplemented with 1.2 $mg l^{-1}$ of 2,4 D and 1 $mg l^{-1}$ thidiazuron (TDZ; MS DT); woody plant medium (WPM; Lloyd and McCown 1980) with 1.2 $mg l^{-1}$ of 2,4 D and 1 $mg l^{-1}$ of KN (WPM DK); and WPM supplemented with 1.2 $mg l^{-1}$ of 2,4 D and 1 $mg l^{-1}$ TDZ (WPM DT). All media contained 3 % (*w/v*) sucrose and 0.7 % (*w/v*) of plant propagation agar (Pronadisa®), in Petri dishes (12 mm ø). The pH was adjusted to 5.7 with KOH (0.1 N) prior to autoclaving for 16 min at 1.1 $kg cm^{-2}$ (122°C).

Culture conditions. The cultures were incubated in a climatic chamber at 25±1°C with two different light regimes. Half of the plates (selected randomly) with the same type and number of explants were kept under 16 h light (45 $\mu mol m^{-2} s^{-1}$, GRO LUX, Sylvania, Surrey, UK) photoperiod and the other half in constant (24 h) darkness.

Experiment design and data collection. Data were collected from 28,080 explants. Four media were tested with 15 explants for each of the 13 types, 6 genotypes, and 2 different light conditions with three replications. During the study, the following parameters were measured:

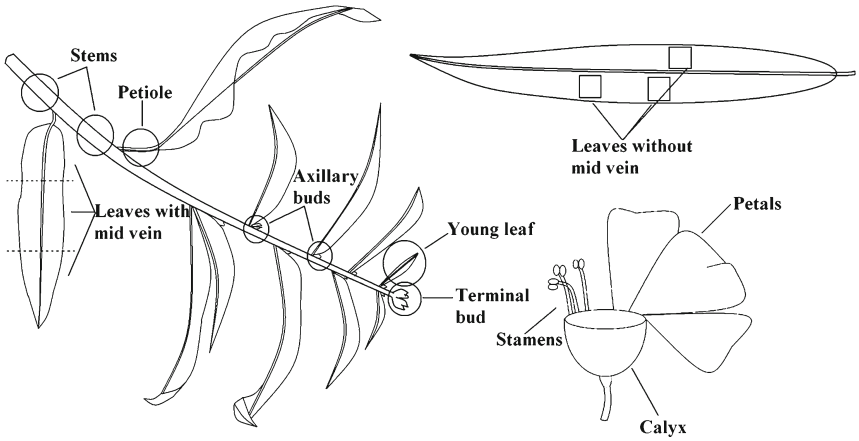

Figure 1. Source of explants used for *in vitro* callus induction in peach.

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percentage of explants in which callus was induced, days to the appearance of callus, color and type of callus produced, and callus relative growth (CRG) which refers to the per centage of explant covered by callus. Data were recorded each day to know exactly when callus started to appear, and at 30 d, CRG was recorded, as well as the number of explants and which part of the explant that the callus growth originated. No subcultures were performed during the ex periment. Significance was determined by analysis of vari ance and the least significance ($P \leq 0.05$) differences among mean values were estimated using Duncan's new multiple range test.

Results and Discussion

Callus was induced in all six genotypes of *P. persica* culti vated on four different media supplemented with 2,4 D, KN, or TDZ and two light regimes: 16 h light and constant darkness. Disinfection resulted in effective prevention of contamination and only 1.4 % of explants died from the process. No significant genotypic differences were observed for any of the three parameters assessed for the eight differ ent treatments (Table 1). A high percentage of callus forma tion was demonstrated for all the six genotypes, where the percentage of explants developing callus ranged from al most 80 % in PS208 to a maximum of 91 % in PS708. Generally, explants started to develop callus from 14 to 16 d after the experiment commenced (Table 1). The amount of callus induced was statistically the same in all the studied genotypes; therefore, further studies in callus induction did not discriminate between peach genotypes.

Thirteen treatments with a variety of explant types were tested for callus induction (Table 2). Statistical differences were observed between groups; however, all the genotypes provided a high level of callus induction for the different explants (above 80 %), and all responded in a similar time frame. The only exception was the stamen explants which were completely unresponsive to all media and conditions tested. Due to the small standard deviation, there were three statistical groups: the stamens and the other two in which the differences between both had a small statistical significance. The lack of response from anthers was unexpected since callus induction from anthers has been reported previously in *Prunus* species. Long *et al.* (1994) and Peixe *et al.* (2004) obtained callus from anthers in *Prunus avium* and *Prunus armeniaca*, respectively. Both authors used culture media different to the ones used in these experiments, with the exception of Peixe *et al.* (2004), who also used MS salts, although their best result was obtained with Nitsch and Nitsch medium. As regards the percentage of explants that produced callus, the rates ranged from 82.37 to 98.96 %, and the average time when the callus started to develop ranged from 14.4 to 17.3 d, similar to the results obtained in almond by Işikalan *et al.* (2010).

Of the 13 explant sources and treatments tested, signifi cant differences were also observed in CRG, which mea sured the percentage of explant covered by callus after 30 d of *in vitro* culture (Table 3). Of the floral tissues, the calyx surface showed the highest CRG (70 96 %). Whereas for the vegetative tissues, the stem treatments (explants plated in either the upright or inverted positions) and the petiole gave the highest CRG. Stem explants plated in the upright position showed surface callus formation covering between

Table 1. Callus induction of six peach genotypes for all four medium and light regimes tested

Cultivar	Explants with callus (%)	Days[z]	CRG[y] (%)
PS708	91.02 a[x]	15.3 a	38.09 a
PS108	84.16 a	15.4 a	38.40 a
PS208	80.96 a	15.8 a	40.28 a
UFO-3®	83.86 a	15.7 a	38.95 a
Early May®	87.12 a	16.4 a	42.36 a
Zise May®	90.04 a	16.1 a	41.29 a

[z] Days of culture required for callus initiation

[y] CRG is the percentage of callus covering the explant

[x] Values in each *column* followed by the same *letter* are not signifi-cantly different ($P<0.05$)

Table 2. Callus induction responses of explant types and treatments for six genotypes of peach

Explant	Explants developing callus (%)	Days to callus formation
Ab-Leaf WV	85.65 b[z]	17.2 b
Ab-Leaf WoV	83.37 b	17.3 b
Ad-Leaf WV	85.52 b	16.3 b
Ad-Leaf WoV	96.44 c	16.6 b
Axillary bud	96.50 c	14.8 a
Calyx	98.96 c	14.5 a
Terminal bud	97.19 c	16.3 b
Juvenile leaf	82.37 b	16.2 b
Petals	97.48 c	16.2 b
Petiole	97.69 c	16.3 b
Stamens	0.00 a	
Stem-up	98.50 c	14.7 a
Stem-in	97.04 c	14.4 a

Ab-Leaf WoV abaxial leaf surface facing the medium without midvein, *Ab-Leaf WV* abaxial leaf surface facing the medium with midvein, *Ad-Leaf WoV* adaxial leaf surface facing the medium without midvein, *Ad-Leaf WV* adaxial leaf surface facing the medium with midvein, *Stem-up* stem placed in an upright position, *Stem-in* stem placed in an inverted position.

[z] Values in each *column* followed by the same *letter* are not signifi-cantly different ($P \leq 0.05$)

t3 1 **Table 3.** Influence of medium, photoperiod, and source of explant on callus relative growth (CRG) of six genotypes of peach

t3 2 Media	MS-DT			MS-DK			WPM-DT			WPM-DK			Average[z]	
t3 3 Photoperiod t3 4 (h of light/24 h)	0	16	Average	0	16	Average	0	16	Average	0	16	Average	0	16
Q5 t3 5 Ab-Leaf WV	32 06	15 64	**23.85**	10 81	11 58	**11.19**	15 89	13 08	**14.49**	61 31	27 11	**44.21**	**30.01 b**	**16.85 b**
t3 6 Ab-Leaf WoV	40 17	8 33	**24.25**	16 39	20 39	**18.39**	15 00	10 33	**12.67**	85 28	21 78	**53.53**	**39.21 c**	**15.21 b**
t3 7 Ad-Leaf WV	41 53	6 67	**24.10**	17 03	25 83	**21.43**	35 17	9 67	**22.42**	56 28	24 88	**40.58**	**37.50 c**	**16.76 b**
t3 8 Ad-Leaf WoV	38 72	24 22	**31.47**	31 39	26 83	**29.11**	45 00	22 33	**33.67**	74 89	36 92	**55.90**	**47.50 e**	**27.58 c**
t3 9 Axillary bud	35 33	53 06	44 19	44 72	37 10	40 91	36 31	29 06	32 68	83 78	35 78	59 78	50 03 f	38 75 d
t3 10 Calyx	80 83	88 00	84 42	**96.00**	91 50	93 75	78 50	72 17	75 33	85 83	**70.00**	77 92	**85.29 j**	**80.42 f**
t3 11 Terminal bud	61 67	52 50	57 08	34 00	25 38	29 69	36 75	43 69	40 22	83 13	41 25	62 19	53 89 g	40 71 d
t3 12 Juvenile leaf	29 33	19 00	24 17	23 96	13 61	18 78	43 47	20 29	31 88	52 50	43 33	47 92	37 32 d	24 06 c
t3 13 Petals	22 50	26 50	24 50	17 67	23 50	20 58	19 00	21 67	20 33	41 67	31 67	36 67	25 21 b, c	25 83 c
t3 14 Petiole	52 67	**42.33**	47 50	60 67	60 50	60 58	48 33	55 33	51 83	**71.67**	57 50	64 58	**58.33 h**	**53.92 e**
t3 15 Stamens	0 00	0 00	0 00	0 00	0 00	0 00	0 00	0 00	0 00	0 00	0 00	0 00	**0.00 a**	**0.00 a**
t3 16 Stem-up	78 56	55 67	67 11	71 31	**30.17**	50 74	67 89	60 17	64 03	**85.56**	49 44	67 50	**75.83 i**	**48.86 e**
t3 17 Stem-in	52 39	**73.67**	63 03	48 78	**25.17**	36 97	61 36	48 33	54 85	57 72	64 50	61 11	**55.06 h**	**52.92 e**
t3 18 Total[y]	**43.52**	**35.81**	**39.67 c**	**36.36**	**30.12**	**33.24 a**	**38.67**	**31.24**	**34.95 b**	**64.58**	**38.78**	**51.68 d**	45 78	33 99

CRG is the percentage of the explant covered by callus

Ab-Leaf WV abaxial leaf surface facing the medium with midvein, *Ab-Leaf WoV* abaxial leaf surface facing the medium without midvein, *Ad-Leaf WV* adaxial leaf surface facing the medium with midvein, *Ad-Leaf WoV* adaxial leaf surface facing the medium without midvein, *Stem-up* stem plated in upright position, *Stem-in* stem plated in inverted position

[z] Values in each *column* followed by the same *letter* are not significantly different ($P \leq 0.05$)

[y] Values in this *row* followed by the same *letter* are not significantly different ($P \leq 0.05$)

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30.17 and 85.56 % of the tissue (similar to the stem in inverted position), while the petiole showed a CRG of 42.33 and 71.67 %. It has been found that the explant source strongly influences the callus induction process. Previous reports have observed the same effect in other species, such as maize (Green and Phillips 1975) and sugarcane (Guiderdoni and Demarly 1988). Also, Declerck and Korban (1996) reported similar findings in peach, as did Ansley *et al.* (2000) for almond explants. In this study, explants consisting of leaves without a midvein presented a higher percentage of callus development than leaves with midvein. It could be due to the fact that leaves without midvein have a greater surface area of wounded tissue, which is known to be more conducive to callus growth.

With regard to the culture media, taking into account all the treatments, WPM salts were the most successful in all the tested explants except for calyx and stem (inverted position) explants, for which, MS salts produced the higher callus induction rate (Table 3). Zhou *et al.* (2010) also found WPM to be more favorable for callus initiation than MS in a regeneration study in peach rootstocks. WPM DK was the most successful treat ment, inducing substantial callus growth in either of the light conditions tested; 64.58 % of the CRG in darkness and 38.78 % under a 16 h photoperiod. MS DT was the second most prolific medium for callus induction, with 43.52 and 35.81 % CRG for 0 and 16 h light, respectively. Therefore, the results show that there is a combined effect induced by the culture medium and the plant growth regulators used.

The callus obtained from explants exposed to the 16 h photoperiod had a green and very compact texture in vege tative tissues (Fig. 2*A C*), whereas in petals (Fig. 2*F*) and calyx (Fig. 2*G*), the calli were mainly pink in color. The

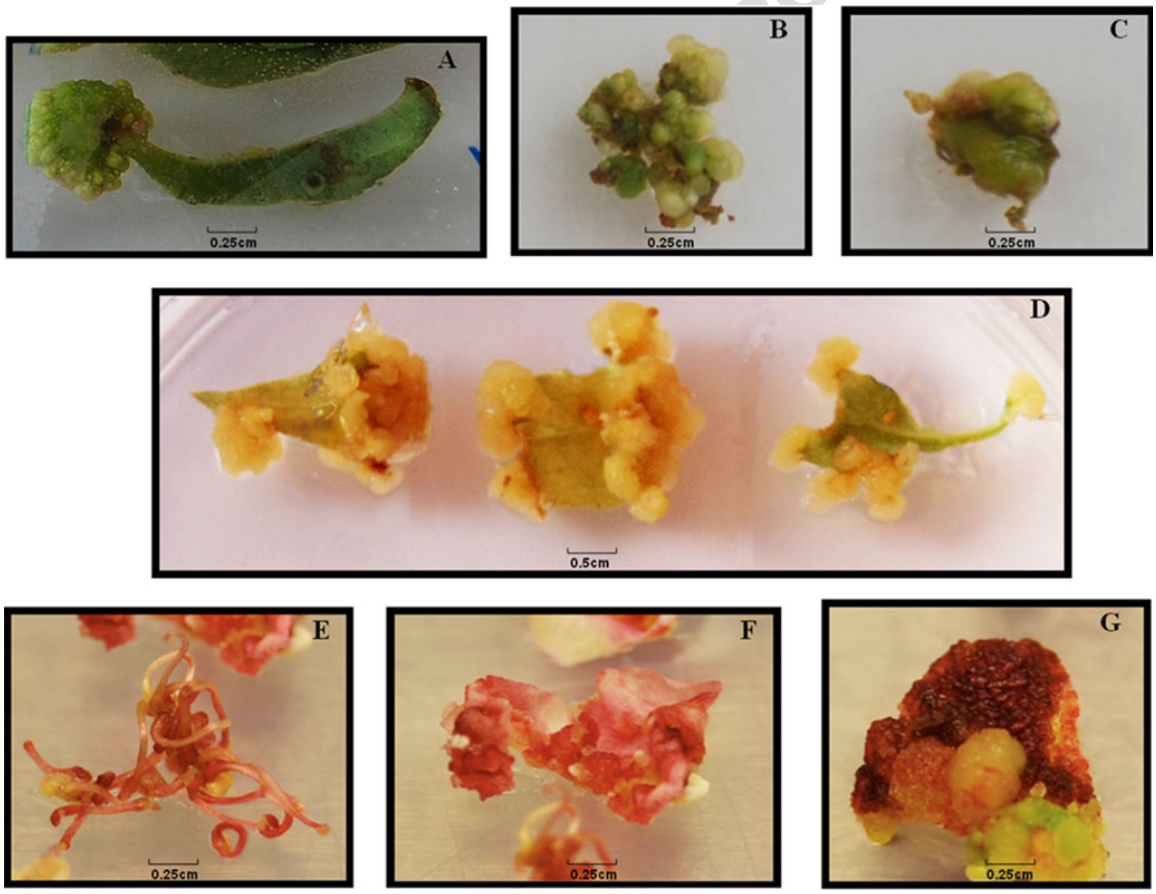

Figure 2. Differences between treatments and explants in callus induction of peach after 30 d. *A* Calli induced in a young leaf under a 16-h light photoperiod. *B* Callus induced in a leaf explant without midvein with the abaxial leaf surface facing the medium under a 16-h light photoperiod. *C* Callus induced in a stem explant plated in upright position under a 16-h photoperiod. *D* Calli induced in leaves with midvein and the abaxial leaf surface facing the medium, in the dark. *E* Stamens with no callus. *F* Callus induced in petals under a 16-h photoperiod. *G* Callus induced in calyx under a 16-h photoperiod.

stamens (Fig. 2*E*) turned pink, but no callus was formed. The appearance of calli in green tissues varied slightly depending on the explant type, but in general, it was composed of small green globular structures (Fig. 2*A*, *B*). In young leaf explants (Fig. 2*A*), the presence of callus was found mainly in the petiole or along the cut surface. These Q6 results agree with the previous research of Zhao *et al.* (2010) where young leaves of the hybrid *P. persica* × *Prunus davidiana* were tested. In this study, under 16 h light conditions, the callus that developed from buds and stems differed from leaves, being limited by the outer structure of the stems (Fig. 2*C*) and buds, while the callus on leaf tissue was unconstrained and globular. The appearance of all calli induced in the dark was the same, regardless of the explant type (Fig. 2*D*) or the culture media. These calli were white to yellow, smooth, friable, and more voluminous than those obtained under light conditions. In all cultures, irrespective of exposure to light, the callus initially developed from cut tissue surfaces. Declerck and Korban (1996) tested different concentrations of auxins (2,4 D, dicamba) and cytokinins (BA, zeatin, kinetin, TDZ) in callus induction of peach. These authors maintained that in leaf tissues of *P. persica*, cytokinins are more likely to produce chlorophyllous and compact callus cultures, whilst auxins increase callus production, inducing friable callus. To the best of our knowledge, those typologies are produced by the light since, in our study, the same media have been used in both conditions and the results were the same in the 13 peach explants, genotypes, or treatments tested. Both typologies of peach callus we observed have been previously described as being embryogenic (white callus; Svircev *et al.* 1993) or organogenic (green callus; Gentile *et al.* 2002; Zhou *et al.* 2010; Pérez Jiménez *et al.* 2012). Friable, white callus can be used effectively in cell suspension cultures due to its propensity to crumble (Bhansali *et al.* 1991).

In summary, this study demonstrates that the growth of *P. persica* calli is greatly influenced by the type of explant, the combination of plant growth regulators, culture media, and light conditions. The media composed of WPM supplemented with 2,4 D and KN induced a higher percentage of callus than the other media tested. A 16 h light photoperiod or constant darkness can be applied for callus induction for different purposes. White to yellow and friable callus was obtained under dark conditions and green compact, nodular callus was produced when explants were cultured in the light. The calyx was the most productive explant with regards to callus induction, followed by the vegetative explants, buds, stems, and petioles. No callus was obtained from the anthers or filaments with the conditions used in this study.

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